Refractory Coeliac Disease

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Refractory Coeliac Disease Diagnosis, Pathogenesis and Treatment

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REFRACTORY COELIAC DISEASE

Diagnosis, Pathogenesis and Treatment

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Table of contents

Part one
Introduction

Part two
Diagnosis

Part three
Pathogenesis

Part four
Treatment

Part five
Summary, discussion and future perspectives

Part six
Addendum

Part one
Introduction

1.
Novel approaches in the management of Refractory Coeliac Disease.

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Summary

Coeliac disease is a gluten-sensitive enteropathy, which commits the patients to a life-long glutenfree diet. This is sufficient to treat the overwhelming majority of patients. However, a small group of these patients, mainly those diagnosed above 50 years of age, fails to improve histologically and clinically upon elimination of gluten from the diet. These patients are regarded as suffering from refractory coeliac disease. In a subgroup of these patients a pre-malignant intraepithelial lymphocyte population can be detected in the small-intestinal mucosa (type II). These patients are at a high risk of developing an enteropathy-associated T-cell lymphoma (50-60% within 4-6 years), which has a very poor prognosis and a 5-year survival of only 8%. The therapeutic challenge in these refractory celiac disease type II patients is targeting the aberrant intraepithelial lymphocytes to eventually prevent enteropathy-associated T-cell lymphoma development. Although management of these patients is difficult and therapeutic options are currently limited, novel treatment modalities are being explored.

Introduction

Coeliac disease (CD) is the most-common food intolerance in the general Western population, with a prevalence of 0.5-1%.[1] Ingestion of wheat gluten leads to chronic inflammation, in genetically susceptible individuals, resulting in villous atrophy and flattening of the small intestinal mucosa. Prompt improvement of nutrient absorption and healing of the characteristic intestinal mucosal lesion is seen upon withdrawal of dietary gluten, which has to be excluded from the diet life-long.

In some patients the intestinal damage can result in malnutrition and severe complications, but only 20-50% of the individuals that are affected have subjective symptoms.[2] CD has long been considered a gastrointestinal disorder of childhood, with classical symptoms, but is now regarded as chronic systemic autoimmune disease more often diagnosed in adults, in which the clinical picture can be very diverse.[3]

The gluten free diet (GFD) is usually sufficient to treat the overwhelming majority of CD patients and clinical improvement is usually evident within the first few weeks after commencing a GFD. However, in some adult patients it may take up to 2 years before a complete restoration of intestinal mucosa is evident.[4] In a small percentage (2-5%) of adult onset CD patients, serious complications develop in the form of refractoriness or development of (pre-)malignant complications. They are regarded as suffering from refractory coeliac disease (RCD) when clinical and histological symptoms persist or recur after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 months, unless earlier intervention is necessary.[5-8] RCD patients are nearly always adults of 50 years of age and over. A relatively high percentage (52%) of these patients develops an Enteropathy-Associated T-cell Lymphoma (EATL) within 4-6 years. EATL is the main cause of death in this patient group, which then has a 5-year survival of only 8%.[9] Early identification of these patients allows for

early therapeutic intervention with a probable significant reduction in morbidity and mortality.[10] However, reliable identification of these patients remains difficult. Diagnostic criteria are depicted in **table 1**.

According to the guidelines of the European Coeliac Disease working group[11], RCD patients can be subdivided into RCD type I and type II patients, with phenotypically normal and aberrant intraepithelial T-lymphocytes (IEL), respectively. IELs are considered aberrant when expressing cytoplasmic CD3ε but lacking surface expression of T-cell markers CD3, CD4 and CD8.[12;13] The presence of these IELs is directly associated with significant risk of EATL development.[6;9;14;15]

In this review, we aim to give an overview of RCD and its pathogenesis, establishing the diagnosis and the available therapeutic options.

The pathogenesis & immunogenetics of (refractory) CD

Pathogenesis

Coeliac disease is an inflammatory condition caused by permanent intolerance for ingested wheat gluten and similar products in barley and rye. It is a multifactorial disease with an interplay between the triggering environmental factor, gluten, the main genetic risk factor, the HLA-DQ2/8 genotypes and the autoantigen: the enzyme tissue transglutaminase (tTG).[16] Adaptive immunity, orchestrated by the lamina propria CD4+ T-cells, has a key role in the gluten-specific T-cell response. The gluten peptides that have passed the epithelial barrier and have been deamidated by tTG are presented by human leukocyte antigen (HLA) class II molecules HLA-DQ2/8 on the cell surface of the antigen presenting cells (APC). Subsequently the deamidated gluten peptides are recognized by CD4+ T-cells and a proinflammatory T-cell response (Th1) is triggered.

In addition, other data suggest that certain parts of gluten (peptide 31-43/49) [17] may induce interleukin-15 (IL-15) production in the intestinal mucosa, which plays a key role in the gluten-induced innate part of the immune system and costimulates the afore-mentioned adaptive response. IELs are activated by IL-15, produced by epithelial cells and macrophages in response to the 'toxic' gluten, resulting in a cytotoxic effector response by these IELs by secretion of interferon-γ (IFN-γ) and expression of the innate immune receptor NKG2D (**Figure 1**). In response to IL-15 and stress, enterocytes upregulate MHC Class I chain-related A (MICA), the MHC-class-I-related epithelial ligand of NKG2D. Binding of this receptor-ligand pair can trigger cytotoxicity of IEL against the epithelial cells, independent of T-cell receptor (TCR) signalling. The interplay between innate and adaptive immunity against gluten orchestrates a progressive destruction of the epithelial layer and expansion of IELs, eventually resulting in villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis. [18;19]

The question remains how RCD patients fail to regain intestinal homeostasis after gluten has been eliminated from the diet. In recent years, studies have pointed towards a key role of the latter innate immune response in this disease process, orchestrated by IL-15. This cytokine has a potent anti-apoptotic effect and specifically induces the expansion and survival of the aberrant IELs that characterize RCD II. Furthermore, the potent pro-inflammatory effect of IL-15 triggers the secretion of

Disease category	Diagnostic criteria	References
RCD I	Villous atrophy persisted or recurred despite strict adherence to a GFD. (assessed by a dietician and negative TGA) At least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria. Excluding other causes of villous atrophy. When ≤ 20% aberrant IELs in intestinal biopsy. IEL phenotype by flow cytometry is normal with the expression of surface CD3, CD4/8 and TCR	[6-9]
RCD II	The same as RCD I, in addition to the presence of ≥ 20% aberrant IEL in intestinal biopsy. The IEL have normal morphology, but exhibit an aberrant phenotype in flow cytometry (lack of surface T-cell markers: CD3,CD4,CD8 and TCR. expression of: surface CD7 and cytoplasmic CD3). EATL has been excluded, as confidently as possible.	[6-9]
UJ	Ulcerations in the jejunum with Marsh IIIA-C in non-involved mucosa. Independent of the duration of the GFD and the % aberrant IELs EATL has been excluded, as confidently as possible.	[60;67]
Secundary EATL	WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues. The patient is already known to have RCD.	[9;59;60]
Primary EATL	WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues. No previous history of complicated celiac disease. Evidence of Marsh IIIA-C in non-involved mucosa.	[9;59;60]

Table 1. Summarizes the diagnostic criteria of the different disease categories

IFNγ by IELs and their cytotoxicity against epithelial cells, thereby promoting ongoing epithelial damage.[20] Uncontrolled overexpression of IL-15 may be the case in complicated CD, in which the highest amounts of IL-15 have been observed,[21] possibly perpetuating epithelial damage and promoting the emergence of the aberrant T-cell population independent of gluten.[20] Also in uncomplicated CD the amount of IL-15 correlated with the degree of mucosal damage.[21] The level at which IL-15 production is deregulated in RCD II and EATL may be either transcriptionally or (post) translationally, however, this remains to be investigated in detail.

T-cell receptor γδ+ IELs may also be involved in the pathogenetic mechanisms of RCD II and EATL, since we have found a relative deficiency of these cells in RCD II patients [22], possibly resulting in a lack of adequate anti-inflammatory TGF-β production with persisting epithelial damage and high IL-15 production. During gluten-withdrawal and subsequent contraction of the TCRαβ+ IEL response, TCRγδ+ IELs may take on an anti-inflammatory role initiating mucosal repair. Evidence for this has recently been provided by Bhagat et al. [23] who have shown that the human TCRγδ+ IELs with the most potent regulatory potential increase upon commencement of a GFD in uncomplicated

CD. Compared with active CD, TCRγδ+ IELs in treated CD showed increased capacity to suppress the cytotoxic arming of CD8+TCRαβ+ IELs via TGF-β production. Further studies are required to establish the exact functional role of TCRγδ+ IEL in the pathogenesis of RCD and EATL development.

Immunogenetics

Coeliac disease is a multigenetic disorder, predominantly associated with the human leukocyte antigen (HLA) class II genotypes, HLA-DQ2 (HLA-DQA1*0501/HLA-DQB1*02) and/or HLA-DQ8 (HLA-DQA1*0301/HLA-DQB1*0302). Most CD patients (95%) carry the DQ2 genotype,[24] encoded either in *cis* or in *trans,* and practically all remaining patients express HLA-DQ8.[25] These molecules represent MHC class II glycoproteins expressed on (professional) APCs. After deamidation of gluten-peptides in the gut by the tissue-transglutaminase enzyme (tTG), improving binding to HLA-DQ2/8 and subsequent presentation by APCs of these peptides to CD4+ T-cells, the T-cell response in the lamina propria of the small intestinal mucosa is initiated.[26] HLA-DQ2 homozygous APCs putatively induce higher magnitude of gliadin-specific T-cell proliferation and cytokine secretion than HLA-DQ2/non-DQ2 heterozygous APCs.[27] This may explain the strongly increased risk for CD development in HLA-DQ2 homozygous individuals,[28] with an earlier onset and more severe disease manifestations.[29;30] In a recent study we found a highly significant correlation between HLA-DQ2 homozygosity and the development of serious complications of CD, in particular RCD II and EATL, implying a gene dose effect.[31] This would indicate that early diagnosis of CD and adherence to a GFD is particularly important for CD patients who are HLA-DQ2 homozygous, as a strict GFD for more than 5 years reduces the risk for malignant complications to that of the general population.[32]

The HLA-DQ2/8 genotypes are necessary but not sufficient to develop the disease. Although almost all CD patients carry HLA-DQ2/8, so does approximately 40% of the healthy Western population.[31;33] Consequently, HLA-DQ2/8 has a high negative predictive value for CD. In patients with (R)CD, the absence of HLA-DQ2/8 is extremely rare. In case a patient is suspected for RCD and the HLA-DQ2/8 genotypes are absent, the initial diagnosis of CD has to be doubted, since it virtually rules out RCD. Especially in case of negative serology before initiation of the GFD, the villous atrophy can probably be attributed to another cause (e.g. the presence of anti-enterocyte antibodies). Importantly, the mere presence of HLA-DQ2/8 only supports the diagnosis, but does not rule out a different cause of villous atrophy, given its limited specificity for RCD.

HLA-DQ2/8 is also a prerequisite for CD in people of heterogeneous ethnic backgrounds.[33] Regarding heritability, twin studies have shown a 75% concordance in monozygotic and 11% concordance in dizygotic twins.[34] Furthermore, the sibling recurrence risk for CD in a British study was 10%.[35] These results imply that, although there is a stronger genetic component in CD than in many other complex diseases, the HLA-genes contribute at most to 40% of the heritable risk and thus HLA genes are not the only causative genetic factor for CD.

So far, the only non-HLA gene identified by positional cloning is the gene *Myosin 9B*,[36] although the effect of this gene could not be confirmed in CD populations in the UK,

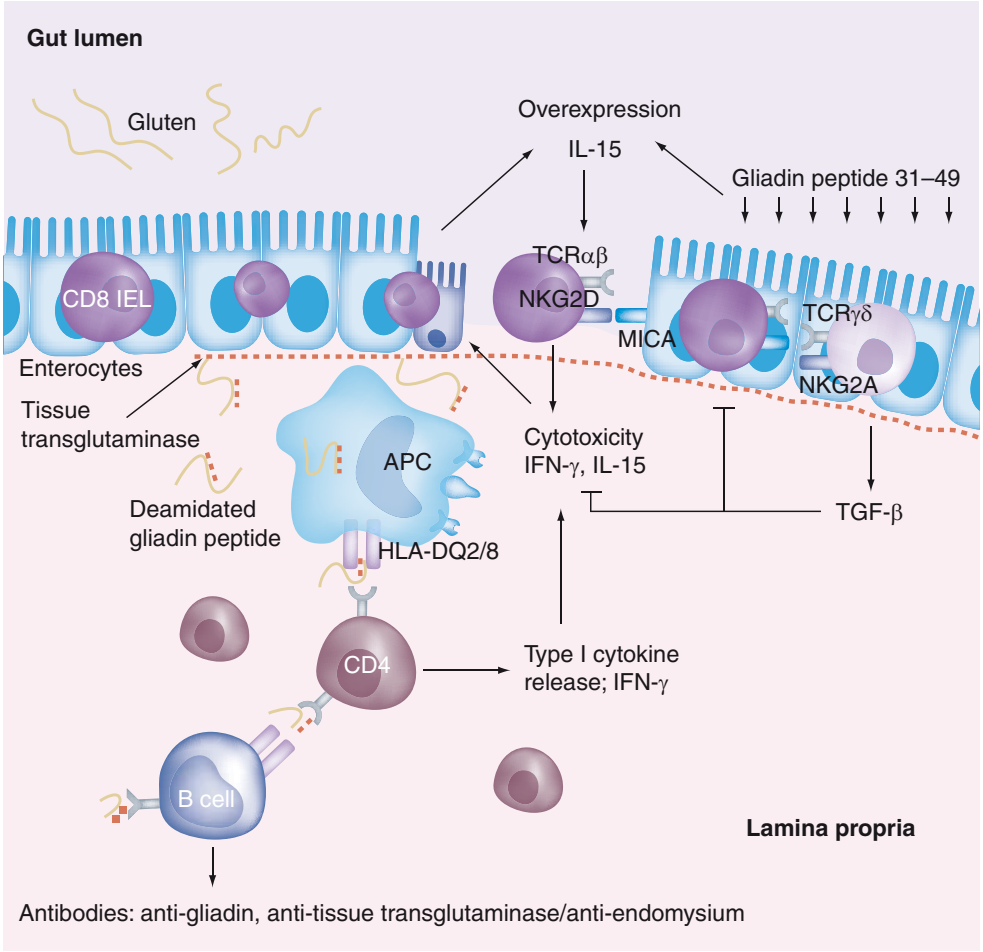

Figure 1. Current view on the immunopathogenesis of coeliac disease. APC: Antigen-presenting cell; IEL: Intraepithelial lymphocyte; HLA: Human leukocyte antigen; TCR: T-cell receptor.

Spain, Italy and Scandinavia.[37-40] The limited replication of *MYO9B* association with CD in other populations reduced the validity of this gene and makes it difficult to interpret the role of *MYO9B* in CD pathogenesis. However, recently positive associations were found between *MYO9B* gene polymorphisms and auto-immune diseases, including CD, in a Spanish cohort[41] as well as the *MYO9B* gene region, CD and dermatitis herpetiformis in a Finnish-Hungarian cohort.[42] In addition, we have recently shown that allelic variants of *MYO9B* also predispose to RCD II and EATL, one single nucleotide polymorphism (SNP) showed a significantly different allele distribution in RCD II and EATL patients compared with controls (p=0.00002). A specific allele was significantly more frequent in RCD II and EATL patients than in CD patients (p=0.0003). This *MYO9B*

SNP predisposed to RCD II and EATL, and increased the risk for these complications in CD patients to a similar extent as and independent of HLA-DQ2homozygosity (odds ratio [OR] 4.3 [95% confidence interval (CI): 1.9–9.8] and 5.4 [95% CI: 3.0–9.6], respectively).[43] Furthermore, a recent genome-wide association study identified risk factors in the region harboring IL2 and IL21, genetic varation in this particular region was suggested to predispose for CD.[44]

Establishing the diagnosis of CD

Intraepithelial lymphocytes in CD

Coeliac Disease is characterized by a permanent increase of TCRγδ+ IELs with a concomitant elevation of infiltrating TCRαβ+ IELs during the active stage of the process.[45-47] However, the TCRαβ+ cells often decrease within months in response to gluten withdrawal, whereas for TCRγδ+ cells this may take years to occur.[47;48] The contribution of TCRγδ+ IELs to the pathogenesis of the villous atrophy in CD still remains unclear. Their persistent increase in CD patients who have recovered a normal mucosa upon a GFD suggests that TCRγδ+ IELs do not induce the epithelial damage directly. This is supported by several studies that showed an increased number of TCRγδ+ IELs in latent CD and dermatitis herpetiformis, in which the intestinal mucosa was still unaffected but progressed to villous atrophy eventually.[49;50] By contrast, the presence of TCRαβ+ IELs correlates with the degree of villous atrophy in CD patients.[47] The increase in TCRγδ+ IELs is considered the only permanent and highly sensitive and specific [51] marker in uncomplicated CD, regardless of dietary treatment and mucosal morphology.[45;46]

Histopathology of the duodenal biopsy specimen

In CD, biopsy of the small intestine remains the gold standard for the diagnosis of CD. In CD, small bowel biopsy specimens show a characteristic, although not specific, mucosal lesion that impairs nutrient absorption by the involved bowel. Histopathological findings can be classified using the modified Marsh criteria for the gluten sensitive spectrum.[11;52;53] The earliest lesion, classified as Marsh I, comprises lymphocytic enteritis with normal villous architecture and marked intraepithelial lymphocytosis (>30 lymphocytes per 100 enterocytes). A Marsh II lesion is present in case of intraepithelial lymphocytosis accompanied by crypt hyperplasia. The majority of CD patients are diagnosed with a Marsh III lesion, which consists of intraepithelial lymphocytosis, crypt hyperplasia and a moderate-to-severe reduction in villous height. This stage can be subdivided in Marsh IIIA with partial villous atrophy, Marsh IIIB with subtotal villous atrophy and Marsh IIIC with total villous atrophy.

Serologic screening for CD

In a recent study the anti-transglutaminase IgA antibodies (TGA) and the antiendomysial IgA antibodies (EMA) proved to be the most sensitive serum antibody tests in a patient population referred for symptoms and signs of CD.[33] Furthermore, in a systematic review of the diagnostic performance of serologic tests for the diagnosis of CD, the

pooled specificity of EMA was close to 100% and of TGA between 95 and 99%.[54] The overall sensitivities were approximately 90%, but the titer correlated with the degree of mucosal damage. As a result, the sensitivity is lower (below 90%) in CD patients with a lesser degree of villous atrophy. The performance of the antigliadin antibodies was inferior to that of EMA and TGA, mainly as a result of their regular presence in healthy individuals and their regular absence in CD. An important pitfall in CD serology is the increased prevalence of IgA deficiency in these patients.[55] In order to avoid false-negative serology in such cases, simultaneous monitoring of either total (nephelometry) or specific (directed against f.i. *E.coli*) IgA levels is required. In case of IgA deficiency serologic screening for IgG antibodies against transglutaminase and preferentially also against endomysium should be performed. Although gliadin antibodies, if present at initial CD diagnosis, are well suited for monitoring the compliance to a GFD, TGA and/or EMA perform equally well.

Establishing the diagnosis of refractory CD

Revision of the initial diagnosis of CD

In a patient with persisting villous atrophy and CD-associated symptoms, refractory to a GFD, the first required step is to reassess the initial diagnosis of CD. The absence of HLA-DQ2/8 genotypes or circulating EMAs/TGAs before the initiation of a GFD strongly suggests an alternative cause of villous atrophy (**Table 2, figure 2**). Although not all CD patients have positive antibodies at presentation, serology tends to correlate with the degree of villous atrophy,[56] and in case of total villous destruction, one usually finds positive antibodies. Furthermore CD is known to be characterized by increased IELs with a concomitant permanent elevation in TCRγδ+ lymphocytes,[49] which may also help to differentiate CD from other diseases.

Evaluating dietary compliance and assessing nutritional status

The main cause for a lack of clinical and histological improvement after initiation of the GFD is dietary mistakes. Considering that a significant number of CD patients (~ 50%) suspected for RCD may indeed have inadvertent gluten ingestion, the dietary compliance has to be carefully reassessed by a skilled dietician and confirmed by negative serology for TGA antibodies (**Figure 2**).[57] The latter usually reverts to negative within 3-6 months on a strict GFD and is strongly indicative for dietary mistakes, whereas, by contrast, EMA can persist for up to 1-2 years.[4]

Importantly, the nutritional status of the RCD patients needs to be assessed by a dietician, as malnutrition is often present, requiring adequate nutritional intervention, adjusted to the needed energy and proteins. In case of intestinal failure, a nasogastric feeding tube or total parenteral nutrition might be indicated. To evaluate the intestinal energy absorption capacity in RCD patients, the single fasting plasma citrulline concentration appears to have poor diagnostic accuracy.[58] Additional research on reliable tests reflecting enterocyte function is warranted to assess nutritional needs and to find biomarkers for permanent intestinal failure, intestinal adaptation and enterocyte repair.

Possible other causes, than CD, for villous atrophy on duodenal biopsy specimens
Villous atrophy with increased IELs
Giardiasis
Postinfectious diarrhea
Tropical sprue
Collagenous sprue
Protein intolerance (cow's milk, soya)
Villous atrophy with (generally) normal number of IELs
Crohn's disease
Tuberculosis
Auto-immune enteropathy
Radiation enteritis
AIDS
Common variable immunodeficiency syndrome
Eosinophylic gastroenteritis
Whipple's disease
Immunoproliferative small intestinal disease

Table 2. Summarizes possible other causes than CD for villous atrophy on duodenal biopsy specimens. Adapted from [5].

Excluding other causes of villous atrophy

The differential diagnosis of small intestinal villous atrophy is extensive (**Table 2**), although some are rare in Western countries, and includes Whipple's disease, Crohn's disease, tuberculosis, radiation enteritis, AIDS, common variable immunodeficiency syndrome, eosinophilic gastroenteritis, autoimmune enteropathy and immunoproliferative small intestinal disease. In the case of villous atrophy with increased numbers of IELs other causes, apart from CD, may be: giardiasis, postinfectious diarrhea, tropical sprue, collagenous sprue and protein intolerance.[5] Before a patient can be regarded as a refractory coeliac, these causes for a failure to improve histologically and clinically upon elimination of gluten from the diet, must be reconsidered.

Flow cytometry of aberrant IELs as a prognostic marker for EATL development

Intraepithelial lymphocytes with an aberrant immunophenotype are known to be a prognostic parameter in RCD and their presence is associated with the development of EATL. Cellier et al. have first shown that RCD is associated with this abnormal subset of IELs of T-cell origin, expressing cytoplasmic CD3ε and restricted rearrangements of the TCRγ chain, but lacking surface expression of T-cell markers CD3, CD4 and CD8.[13] When normal expression of T-cell surface markers occurs (RCD I), the prognosis is less dismal than when an aberrant IEL lymphocyte population is present (RCD II), 50-60% of the latter patients develops EATL within 4-6 years.[9] These EATLs are thought to arise

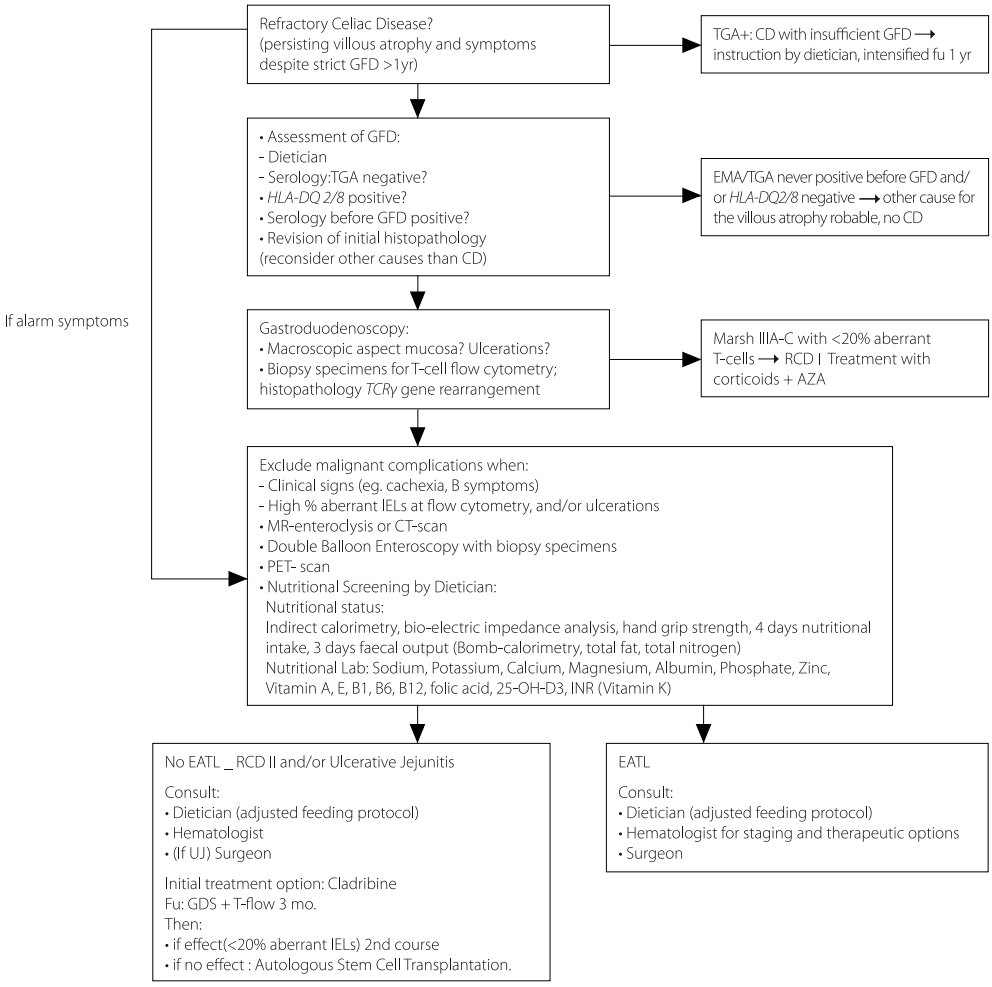

Figure 2. Flowchart for the diagnostic work-up of a patient suspected for RCD or EATL at our center. CD: Coeliac disease; EATL: Enteropathy-associated T-cell lymphoma; EMA: Antiendomysial antibody; GDS: gastroduodenoscopy; GFD: Gluten-free diet; HLA: Human leukocyte antigen; IEL: Intraepithelial T Lymphocyte; Refractory celiac disease; TGA: Anti-transglutaminase antibody; UJ: Ulcerative jejunitis.

from the IEL compartment, with a phenotype comparable to the aberrant IELs in RCD II,[59;60] due to a severe impairment in the intraepithelial homeostasis.[16]

Considering the fact that RCD II patients are at a high risk for development of EATL, accurate discrimination between both types of RCD is of the utmost importance.[9] Diagnostic criteria are listed in **table 1** and the diagnostic work-up in **figure 2**. We have recently defined a cut-off value of 20% aberrant IEL in order to discriminate between RCD I and II, using flow cytometry. IELs were isolated from intestinal biopsies and stained with fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, CD7, CD45, TCRγδ. Cytoplasmic staining of CD3 was performed after cell

permeabilization. Flow cytometric analysis was performed on a standard four-color Fluorescence Activated Cell Scanner (FACSCalibur, BD Biosciences). The data were analyzed using the Cellquest software (BD Biosciences). Care was taken to analyze only viable cellular events based on light-scatter properties. All analyses were performed on lymphocytes, based on bright CD45 staining and low sideward scatter. Aberrant T cells were defined as CD7+ cytoplasmic CD3+, surface CD3, CD4 and CD8 negative cells. The 20% cut-off value has been established based on clinical observations,[9] and via determination of reference ranges for aberrant T-cells in the duodenal mucosa in different CD patient and control groups.[22] In 95% of non-refractory CD patients the highest percentage aberrant IELs was 20%. Using this cut-off value, EATL development was exclusively seen in RCD II patients (median 52% aberrant IELs, range 27-94%). This cut-off value appeared reliable for early risk stratification[9] and targeted therapeutic options in RCD patients.[10;61;62] This is particularly important since, once overt lymphoma has developed, treatment outcome and survival are very poor.[9;63] In addition, quantification of aberrant T-cells is useful for the subsequent follow-up of treated RCD II patients.[10] To estimate whether aberrant IELs are present in the small intestinal mucosa Immunohistochemistry can be performed and a CD3:CD8 ratio can be determined, as described by Patey et al. [12]. However, in tertiary referral centers the benefits of T-cell flow cytometric determination of aberrant IELs are the exact quantification (using a cytoplasmic CD3 staining) of use for follow-up. Prospective comparative studies between the two methods are currently lacking in literature.

Clonality analysis in duodenal biopsy specimens as prognostic marker for EATL development

Regarding T-cell clonality, we have recently compared the presence of aberrant IELs (using a cut-off value of 20%) with the frequently used (pre-)malignant parameter, TCR-γ gene rearrangement, for the predictive value of EATL development in a group of RCD patients.[22] Statistical analysis of our data revealed a much higher negative predictive value and sensitivity (both 100%) for aberrant IELs with regard to EATL development in RCD, when compared to clonality in a duodenal biopsy specimen (75% and 78% respectively). The positive predictive values of these tests for EATL development in RCD were almost comparable (59% for aberrant IELs vs. 50% for monoclonality). However, the majority of the RCD patients with phenotypically aberrant IELs had a monoclonal T-cell population, as shown in this study as well as other studies.[6;13;60]

A study by Daum et al.[64] showed similar results with regard to clonality: a clonal TCRγ-gene rearrangement could be found in the duodenal biopsy specimens of three out of eight patients with a resected EATL, two out of two with UJ, two out of three with RCD evolving to EATL and in one out of six RCD not evolving to EATL, whereas clonal TCR-γ gene rearrangements were present in all EATL specimens.[64]

Excluding small intestinal malignancy

Unexplained weight loss, abdominal pain, fever and night sweating should alarm physicians of an overt EATL. Other markers for overt EATL may be positive stool blood

tests, increased lactate dehydrogenase or beta2-microglobulin. Patients with EATL can present in two different clinical ways (**Table 1**). There are patients with well-established CD who have responded to a GFD but then deteriorate because of the development of RCD II and/or (secondary) EATL. In the other group patients develop EATL without a preceding history of complicated CD and these patients often present with perforation or obstruction (primary EATL).[9] A high index of suspicion for an overt lymphoma should lead to an extensive work-up including upper and lower endoscopy, Ear-Nose-Throat-workup, CT or MRI scan of thorax and abdomen with enteroclysis, video-capsule enteroscopy (VCE) and double balloon enteroscopy (DBE) in order to obtain histological specimens (**Figure 2**).

Enteroscopy using DBE or VCE should be performed in order to search for overt lymphoma. First described by Yamamoto et al.[65] in 2001, DBE is a new endoscopic technique with the potential to allow complete visualization of the entire small bowel and has been proved to be of value in patients with RCD.[66] The finding of mucosal ulcerations, mostly in the jejunum, defines the clinical picture of UJ but may also be present in EATL patients. Sometimes it is hard to differentiate between the two endoscopical features. UJ can be regarded as a 'cryptic' lymphoma.[22;67]

New advances in small bowel imaging can improve the diagnostic accuracy in RCD an/or UJ patients and may be useful to exclude overt lymphoma, these include CT scan and Magnetic resonance enteroclysis (MRE). [68;69] Recently van Weyenberg *et al*.[70] have shown the latter to be a promising tool in discriminating RCD patients with a low and high chance of developing lymphoma, a four-point MRE-scoring system seemed able to identify those patients with (pre-)malignant complications with acceptable sensitivity and specificity and a diagnostic accuracy of almost 90%. This minimally invasive way to evaluate the total small bowel in patients with CD, without radiation exposure, could be of use in deciding which patients should undergo invasive diagnostic procedures such as DBE. Significant differences were observed in RCD II and EATL compared to responsive CD or RCD type I patients, regarding decreased jejunal folds (< 10/5cm), jejunoileal fold pattern reversal, bowel wall thickening and mesenterial fat infiltration. In addition, a splenic volume less than 120 cm^3 was associated with RCD II / EATL.

Furthermore, 18-fluorodeoxyglucose-PET scan has been investigated in a group of patients with EATL and RCD.[71] This modality has been shown to be able to visualize sites affected by EATL, as histopathologically confirmed, in prospective cohort of eight EATL patients and 30 patients with RCD.

However, the exact diagnostic algorithm in RCD remains uncertain, as comparative studies between DBE, PET-CT or MRE and for instance conventional CT-enteroclysis are missing in the literature. Future prospective comparative studies will have to point out the value of these techniques in the work-up of RCD and EATL.

Therapeutic options in RCD

In the past decades different therapies have been evaluated in RCD patients, including conventional corticosteroids, budesonide, infliximab, cyclosporine, azathioprine and

IL-10, but there is no established treatment for these patients yet.[61;72-78] Reports claiming good responses are often difficult to interpret because of the absence of a clear distinction between RCD I and RCD II in these case reports and small series of patients. All treatment modalities that have been evaluated in RCD are listed in **table 3**.

A combination of prednisone and azathioprine is usually sufficient to treat RCD I patients, without aberrant IELs.[61;75] Moreover, no coeliac disease related mortality was observed in a cohort of treated RCD I patients studied, in which the overall 5-year survival was 96%, and no patient with RCD I developed RCD II or EATL within a mean follow-up of 5 years (range 2-15 years).[9] Cellier et al.[6] also reported 3 RCD patients without aberrant T-cells, who made a complete recovery with steroid therapy plus a GFD.

By contrast, all the aforementioned therapies are not successful in RCD II patients with high percentages of aberrant IELs. Results are unsatisfactory and progression to EATL usually occurs despite therapy, possibly even being accelerated by it.[61] This underscores the value of performing T-cell flow cytometry in these RCD patients, since the absence of aberrant IELs in small bowel biopsies at diagnosis of the refractory state seems to indicate a favourable prognosis and conventional treatment with prednisone/azathioprine is usually sufficient. Furthermore, quantification of aberrant IELs is useful for the subsequent follow-up of treated RCD II patients. The aim of therapy in RCD II is targeting of aberrant IEL to eventually prevent EATL development, as this T-cell population determines the risk for the development of EATL in RCD II and these patients are generally regarded as 'cryptic lymphoma' patients.[6;9;14]

Cladribine (2-chlorodeoxyadenosine; 2-CDA), a synthetic purine nucleoside homologue with cytotoxic activity, is the only drug studied thus far showing a significant reduction in aberrant IELs in a large cohort of RCD II patients, although study results were still less than optimal.[62] Furthermore, the theoretical risk of accelerating lymphoma development has to be taken into account, as few cases of secondary malignancies after 2–CDA through T-cell immunodepression have been reported. It has proven valuable in the treatment of hairy cell leukaemia,[79] in which the pathological cells are also CD103 positive as are the aberrant IELs in RCD II. In our recent study, 17 RCD II patients were included and 2-CDA was given intravenously for 5 days (0.1 mg/kg/day), in 1-3 courses every 6 months, depending on the observed response. The drug has a relatively low systemic toxicity profile and was well-tolerated by all patients, without serious adverse side effects. Cladribine therapy might be promising in stabilizing the patient's condition and improving the performance status and the histological picture, as was seen in 58% of the patients. However, it did not prevent eventual EATL development in all patients treated. Seven out of the 17 treated patients (41%) died from EATL. All seven patients had histological improvement of the mucosa, some even with normalization of the villous architecture. Although in some patients a significant decrease in aberrant IELs was observed, they still showed high percentages of these cells after treatment (mean 69%; range 40-91%; standard deviation: 23). In six other patients who did not develop EATL, a significant decrease in aberrant IELs was observed, but only in two patients was the decrease below the 20% cut-off.[22]

Therapy	Type of report	No. of patients	Type of RCD determined	Results	Ref.
Cyclosporine	Case report	1	ND	Remission	[72]
Cyclosporine	Open-Label	13	ND	Clinical and Histological Improvement (61%)	[73]
Interleukin 10	Open-Label	10	ND	Inconsistent Response	[74]
Azathioprine	Open-Label, Prospective	7	Yes	Short-term clinical and histological improvement in 5 of 7 treated patients. Three died (leukopenic fever). Long-term fu. evaluation: 46% mortality rate.	[75]
Prednisone/ azathioprine	Open-Label	10 RCD I, 8 RCD II	Yes	7 of 8 RCD II died (6 from EATL), 10 of 10 RCD I with long-term survival	[61]
Budesonide	Open-Label	4 RCD I, 3 RCD II	Yes	Good clinical and histological response in all RCD I patients, but not in 2/3 of the RCD II patients.	[76]
Infliximab	Case reports	1 RCD I, 1 ND	Yes, ND	Remission, maintenance therapy (prednisone/) azathioprine	[77;78]
Alemtuzumab	Case reports	RCD II	Yes	Clinical Improvement but persisting high aberrant IELs. One patient alive at 9 months fu. the others developed EATL and deceased.	[81;82]
Pentostatine	Case report	1 RCD II / UJ	Yes	Clinical and histological improvement with disappearance of ulcerations and aberrant IELs.	[80]
Cladribine	Open-Label, Prospective	17 RCD II	Yes	Clinical and histological improvement in 58%. No prevention of EATL development in the patients with persisting high % aberrant IELs. (41% died of EATL).	[62]
Autologous Stem Cell Transplantation	Open-Label, Prospective	7 RCD II	Yes	No major non-hematologic toxicity or transplantation-related mortality. Clinical and histological improvement with a significant reduction aberrant IELs. 1/7 died 7 months after transplantation, Autopsy: chronic encephalitis, T lymphocyte infiltration [CMV, HSV negative]. The other 6 good clinical condition at 29 mo. Fu (SD 9.6- range 19-43): 1/6 persistent aberrant IELs with ulcerations. 3/6 M0, 2/6 MI-II. No EATL.	[10]

Table 3. Summary of the treatment modalities evaluated in Refractory Coeliac Disease. Partly adapted from [62]. ND, not determined. mo., months. Fu, follow-up.

Most patients included in the aforementioned study were treated with prednisone and/or azathioprine for months before inclusion. Recently we have started to treat RCD II patients upfront with Cladribine without pretreatment with immunomodulatory drugs. Results so far are more promising, with normalization of the villous atrophy and a significant reduction in aberrant IELs below 20% in all four RCD II patients. Although follow-up is still limited, and it remains to be proven if EATL development can be delayed or even prevented in these patients, this approach appears to be more effective *(Mulder C, personal communication)*.

Pentostatine is another purine analogue inducing T-cell depletion, that is also commonly used and effective in hairy cell leukaemia and chronic lymphoid leukaemia. Only one RCD II / UJ patient treated with pentostatine and budesonide is known, tolerance was good and a dramatic clinical and histological improvement was observed, including not only villous regrowth in the jejunum (although not in the duodenum) but also disappearance of the aberrant IEL population.[80] These results seem very promising but larger series with a longer follow-up period would be needed to evaluate whether it is more effective than Cladribine therapy and is able to prevent EATL development in these patients in the long run. However, unfortunately, since this drug is no longer available such studies are not likely to take place.

Autologous Hematopoietic Stem Cell Transplantation (ASCT) is an increasingly accepted and effective treatment option for patients with severe autoimmune diseases refractory to conventional treatment and has been used successfully in patients with multiple sclerosis, rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus and Crohn's disease. The rationale for this strategy is based on the concept of immunoablation by intense immunosuppression using high dose chemotherapy, with subsequent regeneration of the immune system from reinfused hematopoietic progenitor cells.

High dose chemotherapy followed by ASCT after initial treatment with cladribine might be an alternative approach in these patients with a high risk for development of EATL. Our experience with ASCT in eight patients, after conditioning with fludarabine (40mg/m^2/day orally) and melphalan (70mg/m^2/day iv.), is encouraging in improving the clinical condition but it remains to be established if development of EATL can be delayed or prevented, since follow-up is still limited (mean 15.5 months; range 7-30).[10] No major nonhematologic toxicity or transplantation-related mortality was observed. . In all but one patients, stem cells could be collected by leukapheresis, despite earlier treatment with cladribine. Importantly, there was a significant reduction in the aberrant IELs in duodenal biopsies, after ASCT, which was associated with restoration of villous architecture, although only in two patients the percentage of aberrant IELs was decreased below the 20% cut-off value eventually (the only two with 24 mo. follow-up values). Although these results are promising and this treatment seems feasible and safe for these patients we propose to adjust the protocol for future studies to have complete remission and eradication of the aberrant IEL clone before consolidation with ASCT.

Recently, in several patients we were able to detect aberrant T cell clone(s) in bone marrow, leukapheresis material and even in the peripheral blood, similar to the clone present in the small intestine (unpublished data). Therefore, because of the possible

contamination of the graft by aberrant T cells, we aim to introduce T cell depletion of the graft, using CD34 selection of the leukapheresis material. Furthermore, a more intensive conditioning regimen will be necessary trying to eradicate T cells before transplantation. A combination of fludarabine (intravenously) and alemtuzumab (ant-CD52) as conditioning regimens could be considered, followed by high-dose melphalan for myeloablation before ASCT.

Alemtuzumab (Campath-1H) is a chimeric monoclonal antibody directed against CD52, a T- and B-cell marker. Three RCD II patients treated with this regimen have been described: they all improved clinically but only one was alive after 9-months follow-up (with 30% aberrant IELs left), while the other two patients developed EATL.[81] In one patient treated at our center in a very advanced stage of the disease, the aberrant IELs increased to 91% until EATL developed.[82] A possible explanation may have been that the aberrant IELs were widely disseminated and were not sufficiently targeted by this single-agent antibody, given the fact that, in our patient, almost all aberrant T-cells in the intestinal mucosa still expressed CD52, whereas in peripheral blood barely any (CD52+) lymphocytes could be detected.

However, alemtuzumab has been demonstrated to have clinical activity in a number of T-cell lymphoproliferative disorders, such as T-prolymphocytic leukemia and cutaneous T-cell lymphoma, which are known for their chemoresistance and poor prognosis.[83;84] Single-agent therapy with alemtuzumab does not appear to be curative, but alternative strategies, such as combinations with chemotherapy or consolidation of response with ASCT, may be promising.[84] Therefore, treatment of RCD II with alemtuzumab in combination with chemotherapy and/or ASCT should be explored as it may have additional value in combination with other regimens, to eradicate aberrant IELs in RCD II, before progression to overt lymphoma. Nonetheless, careful monitoring in specialist hematological centers is mandatory, as side-effects of alemtuzumab can be tremendous, including high infectious and haematological toxicities. Furthermore, collaboration by experienced centers is needed in order to set up multicenter trials, pooling data of larger cohorts of these rare patients, to have more power to establish an appropriate treatment.

For future studies, anti-IL-15 may prove to be effective in the treatment of RCD II. Studies implicating a central role for IL-15 in the pathogenesis of RCD II, by orchestrating the cytotoxicity of IELs against enterocytes,[19-21] have suggested IL-15 as a promising therapeutic target in these patients. As mentioned earlier IL-15 is a cytokine with potent proinflammatory and anti-apoptotic properties, which is highly upregulated in the epithelium of patients with RCD II and EATL. In theory, blocking IL-15 might result in healing of the intestinal mucosa and elimination/apoptosis of the aberrant IEL, possibly preventing EATL development. Future trials with a humanized anti-IL-15 antibody in RCD II patients are to be awaited.

Therapeutic options in EATL

Enteropathy-associated T-cell lymphoma is rare, except in the CD population, where the risk has been estimated to be as high as 19.2 times that of the general population.[85]

The annual incidence rate of EATL has been reported to be 0.5-1 per million people in Western countries.[86] The outcome of EATL is very poor with current therapies, with 1- and 5-year survival rates in the range of 31-39% and 11-20%, respectively.[9;87;88] In a prospective multicenter study of 35 patients with EATL treated with six cycles of cyclophosphamide, doxorubicine, vincristine and prednisone (CHOP) chemotherapy, the cumulative 2-year survival was only 28%.[88] Chemotherapy for these patients can be complicated by small bowel perforation, gastrointestinal bleeding and development of enterocolic fistulae.

EATL patients often present at older age (mean:>60 years of age) with advanced-stage disseminated disease, but if EATL is confined to part of the small intestine and if the affected segment (or segments) can be resected, the prognosis might be reasonable; some patients survive more than 5 years. Debulking by surgery might be mandatory, however, prospective studies are lacking in current literature.

It seems that current chemotherapy and high dose conditioning regimens followed by ASCT do not improve the survival in this type of aggressive lymphoma. Relapse regularly occurs within weeks to months after ASCT. Recently, we reported on the feasibility, safety and efficacy of ASCT in four EATL patients after undergoing cytoreductive therapy, including high-dose chemotherapy with or without partial small bowel resection.[63] The patients [(two males, two females, mean age 65 years (range 60-69 years), all stage 4] received ASCT (three patients received upfront transplantation and one was transplanted only after relapse). After partial small bowel resection (three patients), induction chemotherapy and conditioning with BCNU, etoposide, Ara-C and melphalan (BEAM) chemotherapy ASCT was performed. Results showed that all four patients completed the mobilization and leukapheresis procedures successfully and subsequently received conditioning chemotherapy and transplantation. Engraftment occurred in all patients. No major non hematological toxicity or transplantation-related mortality was observed. One patient had ongoing complete remission 32 months after transplantation but three patients died from progressive disease within few months after ASCT.[63] It was concluded that ASCT for patients with EATL seems unsatisfactory. More encouraging results came from a recent report by Bishton et al.[89] describing the treatment of six EATL patients, at an early stage of disease (40-59 years old, stage 1-2), with ASCT after more aggressive chemotherapy, with two cycles of ifosphamide, etoposide, epirubicin, followed by two cycles of high-dose methotrexate (3 g/m^2) with folinic acid rescue and a BEAM. Four patients remained alive in complete remission at 1.8-4.3 years; two relapsed.

Results of ASCT for EATL are, however, still unsatisfactory as patients often present in a more advanced stage of disease. Therefore, earlier diagnosis and the development of more stringent treatments, such as allogeneic stem cell transplantation with reduced intensity conditioning regimen (aSCT-RIC) are urgently required to improve the prospects of these patients, as this is considered a potentially curative treatment option for lymphoma patients in whom conventional treatment has failed.[90] Patients who undergo allogeneic SCT for non-Hodgkin's lymphoma, both indolent and high

grade types, have lower relapse rates than those who undergo ASCT.[91-93] Recently in our center, in an experimental setting the first patient with EATL has undergone allogeneic stem cell transplantation with reduced intensity conditioning regimen.
Alemtuzumab, may also have a role in this setting, because studies have shown improvement in response rates and survival in treated patients with T-cell prolymphocytic leukemia and cutaneous T-cell lymphoma.[83] A recent study in patients with heavily pre-treated peripheral T-cell lymphoma (PTCL) shows that alemtuzumab in combination with CHOP is a feasible chemo-immunotherapy regimen, effective in these patients with a high rate of complete remission achievement, and associated with mostly manageable infectious complications.[94] Therefore, treatment of EATL with alemtuzumab in combination with chemotherapy should be explored. At this time in the Netherlands, alemtuzumab in combination with CHOP chemotherapy is studied in this particular group of patients and results are not available yet.[95] If patients are not suitable for allogeneic SCT, new options, such as a so-called "Sandwich-treatment" with Cladribine, CHOP, Cladribine (C,CHOP,C), could be considered.
In conclusion, it appears that current chemotherapy and high-dose conditioning regimens followed by ASCT do not improve the survival in this type of aggressive lymphoma. Relapse regularly occurs within weeks to months. Therefore, studies instituting therapy at an earlier stage, development of more effective treatments, including alemtuzumab, improving conditioning regimens and possibly the use of T cell-depleted autologous grafts or allogeneic stem cell transplantation with reduced intensity conditioning regimen are urgently required to improve the prospects of these patients.

Key issues

- In a patient with persisting villous atrophy and CD-associated symptoms, refractory to a glutenfree diet (GFD), the first required step is to reassess the initial diagnosis of CD and assess the compliance to the GFD. The absence of HLA-DQ2/8 genotypes or the absence of circulating antiendomysial antibodies/anti-transglutaminase antibodies (TGA) before the initiation of a GFD strongly suggests an alternative cause of villous atrophy. The presence of TGA antibodies after more than 6 months on a GFD antibodies implies continued (inadvertent) gluten ingestion.
- When normal expression of T-cell surface markers is present in Refractory Coeliac Disease patients (RCD I), the prognosis is less better than when an aberrant intraepithelial lymphocyte (IEL) population is present (RCD II); 50-60% of the latter patients develops Enteropathy Associated T-cell Lymphoma (EATL) within 4-6 years, after which the 5-year survival is only 8-20%.
- Quantification of aberrant IELs by flow cytometry is preferable to T-cell clonality analysis for identification of RCD patients at risk for EATL development. A cut-off value of 20% is of use in risk stratification, choice of therapeutic options and subsequent follow-up of RCD patients.
- A combination of prednisone and azathioprine is usually sufficient to treat RCD I patients, in which the overall 5-year survival is 96%, and in our study population no

patient with RCD I developed RCD II or EATL within a mean follow-up of 5 years.

- High dose chemotherapy followed by autologous stem cell transplantation after initial treatment with upfront cladribine may be an effective approach in the RCD II patients with the goal to eradicate the aberrant IEL population and eventually prevent EATL. This procedure is feasible without additional hematologic toxicity, but is only a therapeutic option in selected patients.
- To expand the horizon of the diagnostic and therapeutic arsenal in RCD II and EATL collaboration by experienced centers is needed in order to set up multicenter trials, pooling data of larger cohorts of these rare patients, to have more power to establish an appropriate treatment.

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Scope of the thesis

The current thesis deals with novel developments in *diagnosis* and *treatment*, as well as novel insights into the *pathogenesis* of Refractory Coeliac Disease (RCD). As described in the introduction, a distinction between two types of RCD can be made, of which type II is of risk to develop EATL. However, a clearly differentiating laboratory criterium, based on a validated analytical parameter, has not been postulated for RCD I/II. Only 'the mere presence' of aberrant IELs, revealed by immunohistochemistry, has been reported sofar. Furthermore, the implications of this distinction within the RCD patient group regarding prognosis, as well as subsequent treatment, have remained unclear. Therefore, in this thesis we set out to:
Optimize RCD *diagnosis* by validating a cut-off value for the amount of aberrant IELs in view of accurate distinction between type I and II, using flow cytometry as analytical platform, and to soundly base this with clinical data on the survival of RCD and EATL patients.
Characterize aberrant intestinal T cells in RCD II and indicate other key players involved in RCD *pathogenesis*, at cellular, molecular and genetic levels.
Evaluate novel *treatment* modalities directed at the eradication of aberrant intestinal T cells and/or EATL in order to diminish RCD related morbidity and mortality.

Chapter 1 provides an extensive overview of RCD pathogenesis, involving genetic factors and immunological mechanisms. The work-up in establishing the diagnosis and the subsequent management of RCD is postulated and an outline of the therapeutic options thusfar reported for RCD as well as EATL patients is provided.

In *chapter 2* we set out to gain further insight into the prognosis of RCD and the development of EATL, by reporting on long-term survival and risk of transition of RCD into EATL in a large cohort of patients with complicated coeliac disease.

The epidemiology of EATL in The Netherlands is described in *chapter 3*, by studying the incidence of EATL as well as the demographic characteristics of patients with EATL by searching *the nation-wide network and registry of histo- and cyto-pathology reports* in the Netherlands (abbreviated as *PALGA*)**.** Clinico-pathological data were obtained for 116 cases of EATL, diagnosed between 2000 – 2006.

In *chapter 4* we define and validate the cut-off point between acceptably normal and pathologically increased percentages of aberrant intestinal T cells in RCD. To establish an optimal cut-off value for this percentage, reference ranges for aberrant T cells in the duodenal mucosa of different CD patient and control groups were generated using flow cytometry as analytical platform. Furthermore, the predictive value of this cut-off was compared with intestinal T-cell clonality, as prognostic parameter for EATL development in RCD.

In *chapter 5* we investigated whether aberrant T cells in RCD II could be detected in other parts of the small intestinal mucosa besides the epithelial compartment. In addition, ill-defined skin lesions of RCD II patients were analyzed. Dissemination of aberrant T cells could impose a risk of EATL development outside the intestine. Flow cytometric immunophenotyping was performed on both IEL and lamina propria lymphocyte (LPL) cell suspensions, isolated from fresh small bowel biopsy specimens of RCD II, as wel as on lymphocytes isolated from skin biopsies.

Aberrant IELs and EATL cells are typically cytCD3+, but lack expression of the T cell receptor (TCR)-CD3 complex on the cell surface. It is currently unknown what causes the loss of surface TCR-CD3 expression. In *chapter 6* we report on the generation and molecular characterization of an IEL cell line, derived from a RCD II patient, with the characteristic immunophenotype of EATL. This line was used to explore the underlying mechanism of TCR/CD3 downregulation at the molecular level.

In *chapter 7* we investigated whether TCRγδ+ IELs are decreased in RCD II, providing a possible explanation for persisting mucosal damage and inflammation, and the emergence of aberrant T cells with clonal expansion to EATL. This was hypothesized since TCRγδ+ IELs play an important role in mucosal repair, homeostasis and tumor surveillance. Moreover, recently human small intestinal TCRγδ+ IELs were shown to have regulatory potential in uncomplicated CD.

Chapter 8 describes whether decreased numbers of circulating homeostatic T cells, including Treg, Tγδ and iNKT cells would be associated with the development of RCD or EATL.

The class II human leucocyte antigens (HLA)-DQ2 and HLA-DQ8 loci are the most important predisposing genetic factors in (R)CD identified sofar. HLA-DQ2 homozygosity is associated with complications of CD such as RCD II and EATL. In addition, uncomplicated CD has been linked to genetic variants of the MYO9B gene on chromosome 19. In *chapter 9*, we investigated whether such MYO9B variants are also associated with the development of complicated CD.

Chapter 10 describes the treatment of a RCD II patient with Alemtuzumab (Campath®, monoclonal anti-CD52), used previously for the treatment of mature lymphoma with success. It was evaluated whether intestinal aberrant IELs are sufficiently reached and targeted for eradication by Alemtuzumab.

Autologous Hematopoietic Stem Cell Transplantation (ASCT) is an increasingly accepted treatment for refractory autoimmune diseases. To study the role of ASCT in RCD, *chapter 11* reports on the feasibility, safety and efficacy of ASCT in 7 RCD type II patients, with the ultimate goal of resetting the immune response to prevent or at least delay the development of overt EATL upon transformation of aberrant IEL.

Chapter 12 reports on the feasibility, safety and efficacy of high dose chemotherapy followed by ASCT in 4 patients with EATL (3 upfront and 1 at relapse), with or without prior partial small bowel resection.

A summary of the results described in this thesis as well as future perspectives for research are given in final part of this thesis.

Part two
Diagnosis

2.
Survival in patients with Refractory Coeliac Disease and Enteropathy Associated T-cell Lymphoma: Retrospective evaluation of single centre experience.

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Abstract

Background: Coeliac disease may be regarded as refractory disease (RCD) when symptoms persist or recur despite strict adherence to a gluten-free diet. RCD may be subdivided into types I and II with a phenotypically normal and aberrant intraepithelial T-cell population, respectively. RCD I seems to respond well to azathioprine/prednisone therapy. RCD II is usually resistant to any known therapy and transition into enteropathy-associated T-cell Lymphoma (EATL) is common.

Aim: to provide further insight into RCD and the development of EATL, by reporting on long-term survival and risk of transition of RCD into EATL in a large cohort of patients with complicated coeliac disease.

Design and Methods: Retrospective comparison of responses to therapy in four groups of patients with complicated coeliac disease: 43 RCD-I, 50 RCD II (total), of whom 26 RCD II developed EATL after a period of refractoriness to a gluten-free diet (*secondary* EATL) and 13 were EATL patients without preceding history of complicated coeliac disease (*de novo* EATL). *Results:* No coeliac-disease-related mortality is recognized in the RCD I group. The overall 5-year survival in the RCD I group it was 96%, in the RCD II (total) group was 58% and in RCD II group after developing EATL it was only 8%. The 2 year survival in the *de novo* EATL was 20% versus 15% in *secondary* EATL (P=0.63). Twenty eight (56%) of the 50 patients with RCD II died, 23 (46%) due to EATL, 4 due to a progressive refractory state with emaciation and 1 from neurocoeliac disease.

Conclusion: Remarkably, no patient with RCD I developed RCD II or EATL within the mean follow-up period of 5 years (range 2-15 years). A total of 52% of the RCD II patients developed EATL within 4-6 years after the diagnosis of RCD II. More aggressive and targeted therapies seem necessary in RCD II and EATL.

Introduction

In a small percentage (2-5%) of adult-onset coeliac disease patients refractoriness to a gluten-free diet or pre- and malignant complications develop.[1] Coeliac disease may be regarded as refractory (RCD) when symptoms persist or recur after a former good response despite strict adherence to a gluten-free diet.[1,2,3] We define RCD as persisting villous atrophy with crypt hyperplasia and increased intraepithelial T-lymphocytes (IELs) in spite of a strict gluten-free diet for more than 12 months or when severe symptoms necessitate intervention independent of the duration of the diet.[2,4] Immunologically, two types of RCD are recognized depending on the presence or absence of aberrant IEL in the small-bowel mucosa. When normal expression of T-cell surface markers occurs (RCD I), the prognosis is less dismal than when an aberrant IELs population is present (RCD II).[2,4,5] Patients with RCD II are known to be at a greater risk of developing malignancy, particularly enteropathy-associated T-cell lymphoma (EATL).[2,5,6,7]

There is now strong molecular and immunophenotypic evidence showing that a monoclonal neoplastic T-cell population may emerge from IELs in RCD. Expansion of this T-cell population eventually leads to frank EATL. The genesis and expansion of

these aberrant T-cells involve both inappropriate immune responses to gluten and acquisition of genetic abnormalities. Although the monoclonal IELs in RCD II ca be neoplastic, they are not cytologically abnormal and do not form tumour masses that differentiate these patients from EATL patients, in addition to the absence of radiological and bone marrow evidence of lymphoma. [2,8,9,10]

RCD II is usually resistant to any known therapy that has thus far been tested, including azathioprine/prednisone, cyclosporine and interleukin (IL)-10. [2,11-16] The response to cladribine (2-Chlorodeoxyadenosine) therapy is less than optimal.[17] EATL has a poor outcome with current therapies, with 1- and 5-year survival rates in the range of 31-39% and 11-20%, respectively.[18,19] So far, no systematic analysis of the survival of this group of patients has been reported. The aim of this study is to provide insight into the course of RCD and EATL, by reporting on one of the largest cohorts of patients with complicated coeliac disease in a single centre. We have retrospectively compared the survival in four groups of patients: RCD-I, RCD II total, *secondary* EATL and *de novo* EATL.

Patients and Methods

Patients

We performed a retrospective analysis, providing long-term follow-up data on four categories of patients with complicated forms of coeliac disease in a tertiary referral centre for coeliac disease. From 1992 to 2005 43 with RCD I (12 males and 31 females; mean age at diagnosis of RCD: 49 years, range 23-86 years), 50 patients with RCD II (19 Males: 31 Females; mean age at diagnosis: 59 years, range 47-88 years) of whom 26 patients with *secondary* EATL (11 Males:15 Females; mean age at diagnosis of EATL: 61.5 years, range 52-79 years) and 13 patients with *de novo* EATL (11 Males:2 Females; mean age at diagnosis: 64.3, range 56-72 years) were studied. A small group of patients with RCD have been excluded from the analysis; they were treated with cyclosporine or IL-10. [14, 15]

The baseline characteristics of the patients according to the groups are shown in **table 1**. The patients with RCD I and II were followed for evidence of transition to a more severe state (i.e., the transition from RCD I to RCD II and /or EATL, and from RCD II to EATL) over a mean period of 5 years (range 2-14 years).

Diagnostic criteria

The diagnostic criteria of the different groups are summarized in **table 3**.

In the RCD patients the presence of EATL has been confidently excluded using radiological and endoscopic methods: small bowel follow-through, computed tomography (CT) scanning[23], whole body positron emission tomography (PET) [24], upper gastrointestinal endoscopy, video capsule endoscopy (VCE) and/or double-balloon enteroscopy (DBE) [25] as well as trephine bone marrow biopsies. Those patients diagnosed in 2003 or earlier have negative small bowel follow-through and CT scan, whereas those diagnosed after 2003 have (in addition negative small bowel follow-through and CT-scan) negative PET, VCE and/or DBE.

Evaluation

Clinical, laboratory (haematology, biochemistry and serology), endoscopic and histological examination of the small intestine was performed at regular intervals (3-6 months). Clinically, patients were followed up at the outpatient clinic at regular intervals and their adherence to the diet advised was checked by a dietician. Particular attention was paid to clinical response and adverse effects of therapies.

Antiendomysium antibodies (EMA) and anti-tissue transglutaminase antibodies (anti-tTG) were tested at diagnosis and at follow-up. In all patients, HLA-DQ2/8 typing [28] and flow cytometric immunophenotyping of IELs were performed.

Endoscopy was performed as indicated, using upper gastrointestinal endoscopy,VCE and/or DBE with small-intestinal biopsies.

CT scan, PET scan, magnetic resonance (MR) enteroclysis and dual-energy X-absorptiometry were performed as indicated. The techniques for the VCE, DBE and MR enteroclysis have been available in our centre since the beginning of 2003.

Statistical analysis

Kaplan-Meier survival curves were constructed using SSPS software (SPSS Inc. Chicago, Illinois, USA) for comparison between the groups. Chi square test, odds ratio (OR), log rank and logistic regression tests were used to assess the statistical significance between variables. A p value ≤ 0.05 was considered statistically significant.

Patients Characteristic	RCD-I	RCD-II Total	Secondary EATL	De novo EATL
Total (Male: Female)	43 (12:31)	50 (19:31)	26 (11:15)	13 (11:2)
Age at Dx CD (±SD) (range) in years	47 (±13.5) (21-75)	57 (±6.5) (40-69)	59 (±11.2) (40-69)	64.3 (± 4.5) (56-72)
Age at Dx RCD/EATL (±SD) (range) in years	49 (±14) (23-86)	59 (±9.5) (47-88)	61.5 (±6.5) (52-79)	64.3 (±3.4) (56-72)
Follow-up mean (range) in months	72 (24-240)	44 (8-146)	10 (3 - 50)	12 (3-36)
DQ2 Total -Heterozygous -Homozygous	34 (80%) 23 (54%) 11 (26%)	46 (92%) 23 (46%) 23 (46%)	25 (96%) 7 (27%) 18 (69%)	12 (92.3%) 4 (30.7%) 8 (61.5%)
Aberrant T cells at Dx of RCD and/or EATL mean % (±SD)	3.0 ± 1.9	60 ± 25.9	68 ± 24.4	9 ± 13

Table 1. shows the baseline demographic characteristics received according to the disease group. CD= coeliac disease , Dx= diagnosis, SD = standard deviation

Type of Treatment	RCD I N= 43	RCD II N= 50	Secondary EATL, N= 26	De novo EATL, N= 13	Treatment protocol
Prednisone /topical steroids only	31(72%)	16 (32%)	0	0	Prednisone 40 mg /day 6 weeks, tapered to 10 mg/ day over 6 weeks and, if possible, tapered to 2.5–0 mg daily after 3 months, depending on response
Prednisone + Azathioprine [11]	12 (28%)	34 (68%)*	0	0	Prednisone (as above) + Azathioprine 2 mg/kg/day for ≥ 52 weeks
Prednisone+ Azathioprine followed by 2-CDA [17]	0	23 (46%)	0	0	Prednisone + Azathioprine as above. 2-CDA (0,1 mg/kg/day) intravenously for 5 days, in 1-3 courses every 6 months depending on response
CHOP	0	0	16 (61.5%)	7 (53%)	Standard CHOP 6-8 cycles
ASCT [20,21]	0	6 (12%)	1	3 (30%)	Pretreatment with 1-3 courses of 2-CDA, leucopheresis, preconditioning (Melphalan+Fludarabine) + ASCT
Partial small-intestine resection	0	9 (18%)	6 (23%) *	8 (61.5%)*	-

Table 2. Summary of treatments received, per disease category as defined in table 3. All RCD I, RCD II and secondary EATL have followed gluten-free diet.* P =0.025.

Results

Table 1 shows the baseline demographic characteristics according to the disease group. Regarding gender distribution in the studied groups, there is no difference between the RCD I and RCD II groups. In contrast the *de novo* EATL group showed a statistically significant increase in the female:male ratio compared with the RCD I (P< 0.001) and *secondary* EATL group (P<0.025).

Patients with RCD I were younger than RCD II and EATL patients, but no transition was documented from RCD I to RCD II or EATL during the period of follow-up.

The HLA-DQ2 genotype was present in 80% of RCD I patients, 92% of total RCD II patient, 96% of *secondary* EATL patients and 92% of *de novo* EATL patients. HLA-DQ2 homozygosity is significantly higher in *secondary* EATL and *de novo* EATL compared to RCD I patients, OR= 3,6 (confidence interval (CI) 2,64 - 6,33) and OR=2,06 (CI: 1,88 - 4,78) respectively.[30]

Disease category	Diagnostic criteria	References
RCD I	- Villous atrophy persisted or recurred despite strict adherence to a gluten-free diet. (assessed by a dietician and negative tTGA antibodies) - At least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria - Excluding other causes of villous atrophy. - When ≤ 10% aberrant T-cells in intestinal biopsy. - Intraepithelial lymphocyte phenotype is normal with the expression of surface CD3, CD8 and TCR	2,3,4,8,19
RCD II	- The same as RCD I, in addition to the presence of ≥ 20% aberrant T-cells in intestinal biopsy. - The intraepithelial lymphocytes have normal morphology, but exhibit an aberrant phenotype (normal expression of CD103 and CD7, downregulation of surface CD3 to intracytoplasmic CD3, and the lack of surface T-cell markers: CD4, CD8 and TCR). - EATL has been confidently excluded.	2,3,4, 8,19
Secondary EATL	- WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. - The patient is already known to have coeliac disease or RCD.	26, 27
De novo EATL	- WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. - No previous history of coeliac disease or use of gluten-free diet. - Evidence of Marsh IIIA-C in non-involved mucosa	26, 27

Table 3. Summarizes the diagnostic criteria of the different disease categories

Serologically, all patients with positivity for EMA and/or anti- tTG at the time of diagnosis of coeliac disease reverted to negative after the gluten-free diet, confirming their strict adherence to diet (in addition to clinical assessment by a dietitian). Those with *de novo* EATL have negative serology at the time of diagnosis.

Concerning the mean percentage of aberrant T cells at the time of diagnosis of the RCD and/or EATL, the difference between RCD I (3%) versus RCD II (60%) and between *secondary* EATL (68%) versus *de novo* EATL (9%) was statistically significant ($P < 0.0001$).

Ulcerative jejuntitis was not seen in any patient with RCD I. In the RCD II group, 5 patients of those treated with cladribine had ulcerative jejunitis and 4 of those who subsequently received autologous stem-cell transplantation (ASCT) had persistent histological recovery with disappearance of ulcerations. None of these patients have so far developed EATL. **Table 2** shows the treatments received and summarizes protocols. **Table 3** summarizes the diagnostic criteria of the different disease categories. From all EATL patients, 23 (59%) were treated with chemotherapy (cyclophosphamide, doxorubicine,vincristine and prednisone) (CHOP), whereas the other 16 patients were not eligible for chemotherapy due to a bad general condition at the time of diagnosis. Fourteen (36%) patients underwent partial resection of the small intestine. Laparotomy was needed in 3 patients to establish a diagnosis and 11 were operated for complications

	RCD-I N= 43	RCD-II (total) N= 50	EATL (RCD II) N= 26	De novo EATL N= 13
EATL	0	23 (46%)	23 (88.4%)	9 (69%)
Refractory state and emaciation	0	4 (8%)	0	0
Other coeliac- related	0	1 neurocoeliac	0	
Unrelated	3 (6.9%) (1 alcoholic cirrhosis, 1 COPD, 1 lung carcinoma)	0	0	0
Alive	40 (93.1%)	22 (44%)	3 (11.6%)*	4 (21%)*

Table 4. Causes of death according to the patients categories. * P value= 0.63

(5 for perforations and 6 for obstructive symptoms). Seven patients were treated both by chemotherapy and resection. Eight patients (61.5%) of the *de novo* EATL group underwent partial small-intestine resection compared with only 6 (23%) patients of the *secondary* EATL group (P= 0.025). Three other RCD II patients had surgery because of ulcerative jejunitis with perforations. Four patients with EATL were treated with high-dose chemotherapy followed by ASCT (3 with *de novo* EATL and one with secondary EATL) and 3 of them died within few months thereafter.[21]

Table 4 shows causes of death according to patients' categories. In the RCD I group, only 3 patients died during follow-up, all of them from unrelated illnesses. In the RCD II group, 26 patients (52%) developed EATL within 4-6 years after the diagnosis RCD II had been made, 23 RCD II patients (46%) died after developing EATL. Four (8%) patients died due to progressive malabsorption with emaciation; one patient developed extensive multifocal squamous-cell carcinoma of the skin (> 15 lesions). One patient died due to progressive neurocoeliac disease 8 months after ASCT.[20] Nine of 13 (69%) patients with *de novo* EATL and 23 of 26 (88.4%) patients with *secondary* EATL died within months of diagnosis.

Figure 1 (**A and B**) shows the Kaplan-Meier curve of survival according to the disease group. The 5- year survival was 96% in RCD I versus 58% in RCD II (total) (P= 0.001). On the other hand, the 2-year survival in the *de novo* EATL group was 20% versus 15% in the EATL (RCD II) group (P=0.63). Interestingly, the most significant drop in survival in these groups was observed in the first 2 years after diagnosis. The longest survival thus far in the de novo EATL was 26 months.

As this is not a randomised study, it is not possible to make definitive conclusions about the success of different treatments. However, in the prednisone-alone group, the 5 year survival was 25%; in the prednisone and azathioprine group 36% (P= 0.43); and in the cladribine (2-CDA) group 22% (43 % at 36 months); P=0.97. With respect to EATL development, there was no statistically significant difference between the groups.

Engraftment occurred in all RCD II patients who received ASCT. Neither major non-haematological toxicity nor transplantation-related mortality was observed. The mean follow-up duration was 16 months (range 8-31 months). Within 3-4 months of ASCT all patients had normalisation of stools frequency, disappearance of abdominal pain and improvement in biochemical markers. In addition, improvement of the body mass index, serum albumin, endoscopical findings and histology was documented. One patient with preexistent neurocoeliac disease mimicking multiple sclerosis developed progression and died 8 months after transplantation. The details of the protocol, inclusion and detailed results are described in our recent article.[20]

Discussion

The development of a refractory state in patients with coeliac disease may herald the start of a very serious phase in the evolution of the disease state - particularly in RCD patients with aberrant T cells (RCD II). [1, 2] Particular attention should be paid to detect non-compliance or inadvertent gluten ingestion. Persistent positive serology may point to this latter mentioned scenario. [31-33]

Regarding gender distribution in the studied groups, there is a female:male predominance in the RCD I, RCD II total and *secondary* EATL group, whereas there is a reversed ratio with more males than females in the *de novo* EATL group. The difference is not significant between the RCD I and RCD II groups (p= 1.05). However, it is highly significant in the *de novo* EATL versus RCD I (P< 0.001) groups and versus the *secondary*

Figure 1 A

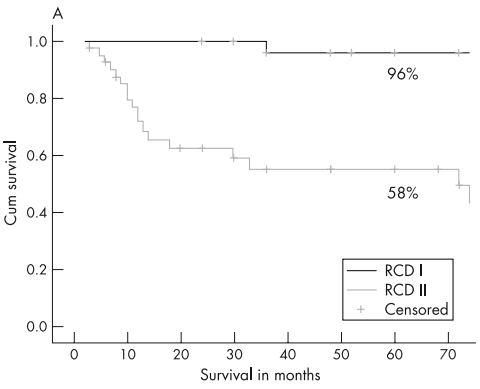

Figure 1 B

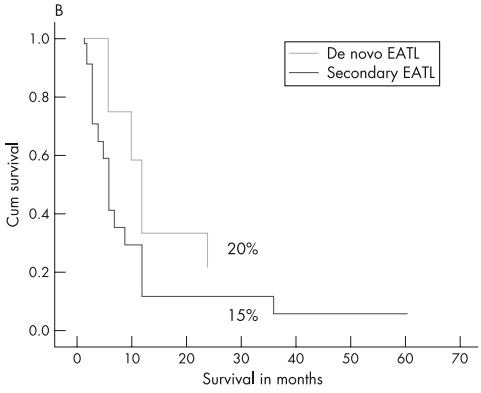

Figure 1. Shows the Kaplan – Meyer curve of survival according to the disease group. (**A**) The 5- years survival is 96% in the RCD I group versus 58% in RCD II (P= 000.1). (**B**) The 2 year survival is 15% in secondary EATL versus 20% in de novo EATL (P=0.63). Censored= Not lost for follow-up

EATL group (P<0.025). Other studies also reported a predominance for EATL in males, with a peak in the 6th decade of life [7, 33, 34]; however the majority of patients with uncomplicated coeliac disease are female.[35, 36]

HLA-DQ2 homozygosity is significantly higher in the *secondary* EATL and *de novo* EATL groups compared with the RCD I group, OR= 3,6 (CI: 2,64 - 6,33) and OR=2,06 (CI: 1,88 - 4,78), respectively. As we reported earlier, there is an association between DQ2 homozygosity and complicated forms of coeliac disease.[30]

A combination of prednisone and azathioprine is usually sufficient to treat RCD I patients.[11, 16] None of our RCD I patients has progressed to RCD II during follow-up. Cellier et al.[2] also reported 3 RCD patients without aberrant T-cells, who made a complete recovery with steroid therapy plus a gluten-free diet. This underscores the value of performing T cell flow cytometry in these patients, as the absence of aberrant T cells at diagnosis of the refractory state seems to indicate a favourable prognosis and conventional treatment with prednisone with or without azathioprine is usually sufficient. In view of the poor prognosis of EATL, the question has always been: can the diagnosis of lymphoma be made earlier to allow more effective treatment, thereby improving the prognosis? Despite state-of the-art technologies used, we can only assume that lymphoma has been excluded in these RCD II patients. The question remains as to whether a "subclinical" lymphoma was actually present and/or its development can be accelerated by available therapies.[37]

Different therapies have been evaluated, but there is no effective therapy available yet for these RCD II patients yet.[2, 11-17] Reports claiming good response are difficult to interpret due to an absence of clear distinction between RCD I and RCD II in these series. Cladribine (2-CDA) therapy might be promising in stabilizing a patient's condition, and improves the performance status and the histological picture as seen in 58% of our group. However, it does not prevent EATL.[17] Nine patients (39%) of 23 treated patients died from EATL. High dose chemotherapy followed by ASCT after stabilisation with cladribine might be an alternative approach in these pre-lymphoma patients. Our experience with the first 7 patients is encouraging, but it remains to be proven if development of EATL can be delayed or prevented.[17]

Factors that seem to be associated with a high risk for EATL development in coeliac disease are old-age presentation, male sex, ulcerative jejunitis, presence of aberrant T cells and DQ2 homozygosity.[30,39,40] However, we have seen disappearance of the ulcerative lesions after cladribine therapy and/or ASCT in five patients, and none of them has developed EATL so far.[17] Thus, in case of early intervention in the high risk RCD II group, specifically ulcerative jejunitis - EATL development might be prevented.

Patients with EATL can present in two different clinical patterns. There are patients with well-established coeliac disease who deteriorate because of the development of RCD II and eventually develop *secondary* EATL. In the other group patients develop EATL without a preceding history of complicated coeliac disease, these patients often present with perforation or obstruction (primary or *de novo* EATL). The aberrant T-cells in primary EATL patients appear to be largely confined to the tumour mass and cannot be found in such high percentages diffusely throughout the small intestine as in secondary EATL patients (P<0.0001), possibly suggesting a different pathogenesis pathway.

Nine of 13 (69%) of *de novo* EATL patients and 23 of the 26 *secondary* EATL patients (88.4%) died despite therapy. The 2-year survival in the *de novo* EATL group is 20% versus 15% in the EATL (RCD II) group (P=0.63). Interestingly, 61.5% of the *de novo* EATL group had undergone resection compared to 23% of *secondary* EATL (P= 0.025). Howdle et al [7] reported a laparotomy rate of 73% in lymphomas associated with coeliac disease. It may be necessary to resort to laparotomy when lymphoma is suspected and when the diagnosis can not be established with less invasive methods. Overall, surgery was needed in 3 patients to establish a diagnosis and 11 had surgery for complications (5 for perforations and 6 for obstructive symptoms). Surgery, radiotherapy and chemotherapy may be used depending on stage and clinical condition. EATL is often disseminated at diagnosis and has almost always a dismal outcome. However, if EATL is confined to part of the small intestine and if the affected segment (or segments) can be resected, the prognosis might be reasonable; some patients survive more than 5 years.[41,42] Debulking by surgery might be mandatory; however prospective studies are lacking. Three of 4 EATL patients who received high-dose chemotherapy and ASCT died within months after transplantation (data submitted).

Although the studied groups of patients seem to be rather heterogeneous and not entirely exclusive, we think that we provided here a detailed description of the prognosis and response to currently available therapeutic options in the whole spectrum of complicated forms of coeliac disease - ranging from RCD I (with its relatively benign course) to RCD II (which has a definite pre-lymphoma potential to the frankly malignant EATL). Furthermore, this group of patients is one of the largest cohorts reported from a single centre dealing with coeliac disease and its complications. The most important papers dealing with RCD described series ranging from 1-16 RCD II patients. [2, 8, 22, 29, 43-46]

In conclusion, an extensive evaluation and aggressive targeted management might be helpful in dealing with complicated forms of coeliac disease. Studies are needed to define more precisely the cut-off point between acceptably normal and pathologically increased percentages of aberrant T cells. Multicentre cooperation and studies are required to further increase the understanding of RCD in general.

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3.
The incidence of Enteropathy Associated T-cell Lymphoma: a nation-wide study of a population-based registry in the Netherlands.

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Abstract

Objective: Enteropathy-associated T-cell lymphomas (EATLs) are T-cell non-Hodgkin lymphomas of the small bowel, which are specifically associated with coeliac disease (CD). To our knowledge no studies have previously reported on the overall incidence of EATLs in the general population. The aim of this study was to investigate the incidence of EATL and the demographic characteristics of patients with EATL in The Netherlands.
Material and methods: A survey of the *nation-wide network and registry of histo- and cyto-pathology reports in The Netherlands (PALGA)* was performed. We included all T-cell lymphomas detected between January 2000 – December 2006 that initially presented in the small bowel. Crude and world standardized incidence rates were computed as well as gender- and age-specific incidence rates. Finally, the distribution of characteristics such as the localization, the Marsh classification and method of diagnosis are described.
Results: Clinicopathological data were gathered for 116 cases of EATL. The mean age at primary presentation of EATL was 64 years. The crude incidence in the total Dutch population was 0.10/100,000 with an incidence of 2.08/100,000 over the 50-year-olds. Age-specific incidences were 1.44/100,000 in the 50-59 years age group, 2.92/100,000 in the 60-69 age-group, and 2.53/100,000 in the 70-79 age-group. There was a significant predominance of males (64%, p=0.004, CI 54-72); above the age of 50 the gender-specific incidence was 2.95/100,000 in males versus 1.09/100,000 in females. Most EATL's were localized in the proximal small intestine and the diagnosis was made by surgical resection in the majority of cases.
Conclusions: EATL is a rare disease with an incidence of 0.10 per 100.000 inhabitants per year, occurring in older age, with a peak incidence in the 7th decade. The tumour is mainly localized in the proximal small intestine. Although uncomplicated CD is twice as frequent in female patients, EATL and is more prevalent in males.

Introduction

Enteropathy-associated T-cell Lymphoma (EATL) is a rare form of aggressive T-cell lymphoma, estimated to have an annual incidence rate of 0.5-1 per million people in Western countries.[1] It has a poor outcome with current therapies, with survival rates of 1, 2 and 5 years in the range of 31-39%, 15-20% and 8-20%, respectively.[2-4] Despite the rarity of this type of lymphoma, it is one of the main causes of death in patients with coeliac disease (CD) diagnosed in adulthoud.[5] CD is the most common food intolerance disorder in the general Western population, with a prevalence of 0.5-1%.[6] In patients with CD, ingestion of wheat gluten, which has to be excluded from the diet life-long, leads to chronic inflammation of the small intestinal mucosa, resulting in intraepithelial lymphocytosis and villous atrophy. In some patients, the intestinal damage can result in malnutrition and severe complications, such as osteoporosis and EATL, but only 20-50% of the individuals that are affected have evident symptoms.[7] CD has long been considered a gastrointestinal disorder of childhood, with classical diarrhoea-

predominant symptoms and a malabsorption syndrome. At present, however, it is regarded as a chronic systemic autoimmune disease which is more often diagnosed in adults. The clinical picture can be diverse and consist of non-specific complaints, which may delay actual diagnosis. [8]

After several reports on small intestinal T-cell lymphomas as a complication of adult CD, the first being as early as 1962,[9;10] the term "Enteropathy-Associated T-cell Lymphoma" was introduced by O'Farrelly et al.[11] It has now been incorporated in the WHO classification of tumours of haematopoietic and lymphoid tissues as enteropathy-type T-cell lymphoma, the only clinicopathologically defined primary gastrointestinal T-cell lymphoma.[12] In the past few decades a number of studies have been conducted to quantify the magnitude of the cancer risk in CD patients. In two studies, in which large cohorts of non-Hodgkin lymphoma (NHL) patients were serologically screened for CD and compared with controls, CD was indeed found to be associated with an overall increased risk for developing NHL (odds ratio (OR) 2.6 - 3.1; 95% confidence intervals (CIs) 1.4 - 4.9 and 1.3 - 7.6 respectively), in particular EATL (OR 28, 95% CI 6-144).[13;14] Clinically silent CD was rare in patients with EATL and the diagnosis of CD preceded the onset of EATL in most cases.[13] The increased cancer risk in CD patients was not judged sufficiently large to justify early mass screening for CD. Instead, it was suggested to search actively for the presence of CD only in patients with a T-cell lymphoma and in all lymphomas with a primary gut localiszation[13;14], as EATLs are almost exclusively observed in CD patients.[14;15]

Other studies, monitoring malignancy and mortality within CD patient-cohorts, indicated an up to a 2-fold increased mortality risk in CD, and a 69-fold increased risk of dying from lymphoma. Thereby, intestinal lymphoma was the main cause of death in adult CD patients.[5] Interestingly, the increased mortality risk was mainly evident within 3 years after the diagnosis of CD and was dependent on the delay in diagnosis of CD (>120 months), poor adherence to a glutenfree diet (GFD) and the severity of CD-related symptoms at presentation.[14] Askling et al.[16] have shown that only adults, but not CD patients diagnosed durin childhood and adolescence, had an elevated cancer incidence. Strict adherence to a GFD, especially if started during the first years of life, seems to play a protective role in the development of this rare but very aggressive form of cancer.[1;17]

Whereas the risk for malignancy and mortality in CD has been extensively evaluated, both in a population-based as well and in a referral-centre setting, the magnitude of the incidence of EATL, as well as its demographic characteristics, has never been established through a national survey. To address this, we have carried out a search in *the Nationwide Network and Registry of Histo- and Cytopathology in the Netherlands* (abbreviated as *PALGA*), which is a central database for all histopathological diagnoses in the country.[18]

Design and Methods

The PALGA registry

The PALGA database is a central archive containing the abstracts of all histopathological and cytological reports from all hospitals in the Netherlands since 1991.[18] Every record contains encrypted patient characteristics, a summary of the pathological

findings and diagnostic codes similar to the Systematized Nomenclature of Medicine, according to the classification of the college of American pathologists (SNOMED).[19] Currently, the database contains about 42 million records of approximately 10 million patients.

Design of the study

The present study was based on the data recorded in the PALGA registry between January 2000 and December 2006. EATL was defined as T-cell lymphoma with a tumour initially presenting in the small bowel, irrespective of the presence of CD. Excluded were ALK-positive anaplastic large-cell lymphomas and nasal type NK/T-cell lymphomas, because these lymphomas are thought to have a CD-unrelated pathogenesis.[12]

The following terms were used in the search: Small bowel, small intestine, malignant lymphoma, enteropathy associated T-cell lymphoma.
From all patient data obtained, we selected patients with the combination EATL or T-cell lymphoma and CD, including all T-cell lymphoma biopsy or resection specimens. The records were checked for reports of specific anatomical subsites in the small intestine (duodenum, jejunum, ileum) and the noted presence of features associated with CD; such as enteropathy, gluten-sensitive enteropathy, sprue and the grade of villous atrophy according to the modified Marsh classification.[20;21] Data were corrected for repeated histological specimens within the same patient and entries resulting form revisions of histological EATL specimens obtained before January 2000.

Data analysis

Crude overall and age-specific (10 year age groups) incidence rates were computed using the Dutch population structure. Standardised incidence rates were computed by weighing the age-specific incidence rates with the WHO world population structure.[22] To establish gender differences, we also computed the incidence for men and women separately. Finally, we report on the method of establishing the diagnosis (endoscopic, surgical resection, autopsy or a combination of these), the localization of the tumour and whether or not an association is mentioned with the presence of CD. For the analyses, we used SSPS software (version 11.0; SPSS Inc., Chicago, Ill.,USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Between January 2000 and December 2006, a total of 889 entries of small bowel lymphoma were found. All entries are depicted in the pie chart in **figure 1**. EATL was found in 116 patients (74 M, 42 F). The remaining 773 patients were diagnosed with different types of B-cell lymphoma (n=717), NK-T cell lymphoma (n=1) and ALK-positive anaplastic large cell lymphoma (n=3). Fifty-two lymphomas were not specified as B- or T-cell type, but simply as "malignant lymphoma".

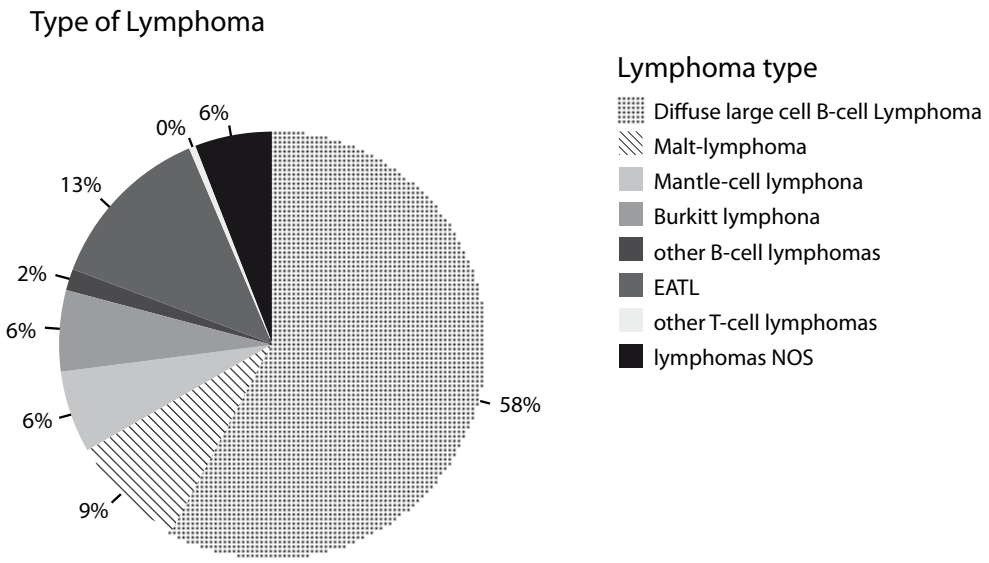

Figure 1. The type of primary malignant lymphoma in the Netherlands from 2000 to 2006

The number of EATL patients in each age group and the gender distribution are presented in **table 1**. The mean age at diagnosis of EATL was 64 years (range 33-92, SD=11) and 92% of the patients were over 50 years of age. Significantly more EATL patients were male (64%, CI: 54-72%, p=0.004). The crude incidence in the total Dutch population was 0.10/100,000 inhabitants. In the Dutch population aged over 50 years, the incidence was 2.08/100,000 inhabitants. Age-standardised incidence rates were 0,50/100,000 in the total population and 2.09/100,000 above 50 years of age. Age-specific incidences as well as gender-specific incidences are shown in **figure 2**, per 10-year age-group. In males, the overall crude incidence was 0.13/100,000, with an incidence of 2.95/100,000 in men over 50 years of age. In women, the overall crude incidence was 0.07/100,000, and 1.09/100,000 above 50 years of age.

Figure 3 shows the localization of the EATL in the histological specimens registered in *PALGA*. EATL was mostly localized in the proximal small intestine, 39% in duodenum and jejunum compared with only 9% localized primarily in the ileum, while 6% was localized multifocally. No specific localization, other than "small intestine", was provided for 47% of patients. Furthermore, the procedure by which the histological specimens of EATL from the PALGA registry were obtained was mostly surgery (81%). Sixty-eight percent of the EATL patients underwent surgery with resection of the EATL, and some for full-thickness biopsy without resection. Nineteen percent of the patients had a biopsy of the EATL by endoscopy, and in 13% of the cases both endoscopy with biopsy and surgical resection were performed.

In some patients (11/116) a documented (biopsy-proven) history of CD was mentioned in the histopathological report, preceding the development of lymphoma. Other reports mentioned histological features consistent with CD in resected areas of the small bowel not infiltrated with lymphoma. In 30% of the EATL patients the Marsh classification was specified (see **table 2**). Twenty percent of the 116 EATL records mentioned 'consistent with coeliac disease', but no exact grade of villous atrophy. The remaining 53% of the records did not mention CD, but only referred to "Enteropathy-associated" T-cell lymphoma.

Patient characteristic Age (yrs)	No.	(%)
<50	9	(8)
50-59	31	(27)
60-69	42	(36)
70-79	26	(22)
>80	8	(7)
Sex		
Male	74	(64)
Female	42	(36)

Table 1. Age distribution and gender of all 116 EATL patients in The Netherlands from 2000 to 2006.

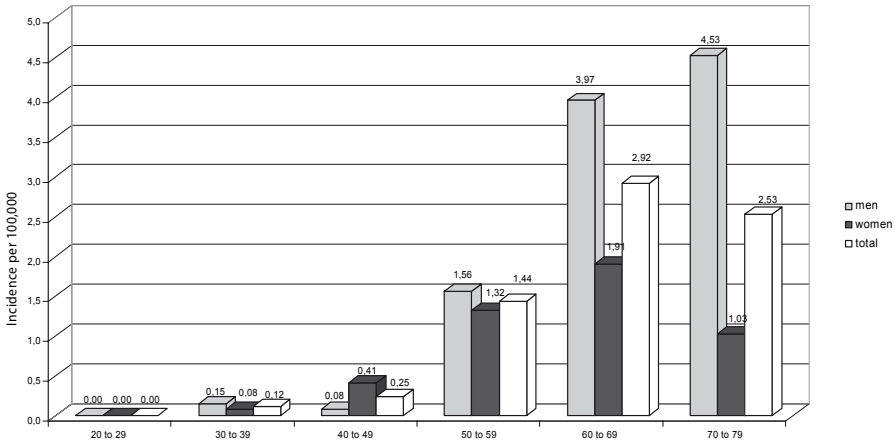

Figure 2. Age-specific incidence of enteropathy-associated T-cell lymphoma (EATL) per 100.000 inhabitants in the Netherlands, for 10-years age groups.

Discussion

In this study, we estimated the incidence of EATL in The Netherlands based on the registration of cases in *the Nation-wide Network and Registry of Histo- and Cytopathology (PALGA)*. There were more male than female EATL patients (64% versus 36%) and the crude incidence in the total Dutch population was 0.10/100,000. In the population over 50 years of age, the incidence was 2.08/100,000. Highest age-specific incidence was observed in the 7th decade of life (2.92/100,000). These findings are in accordance with previous reports, in which EATL occurred exclusively in elderly CD patients.[2;23] Sofar, most studies on EATL have been conducted in referral centres and CD patient cohorts, and to our knowledge no population-based studies on the incidence of EATL have been reported previously. However, several earlier population-based studies on the incidence of extra-nodal lymphomas in the small bowel in general, both T- and B-cell NHL, have been conducted. These studies reported age-standardised annual incidence rates in the range of 0.17/100,000 – 0.48/100,000.[24;25] In all studies a higher incidence in males was observed for intestinal NHL at all ages; but all patients were mostly limited to a population aged over 50 years.[24;26] Furthermore, what is remarkable is that not only was intestinal NHL more frequent in males, it also had a higher occurrence of disseminated disease, high-grade histology and T-cell phenotype.[25]
EATL patients were also predominantly male in the present study, with an incidence above the age of 50 almost three times as high as that in females (M:2.95/100,000 versus F:1.09/100,000). This is also confirmed by a previous study evaluating survival in a cohort of patients with complicated CD from our institution[4] in which we found a predominance of male EATL patients. These were especially patients without a known history of CD (*de novo* EATL, M:F, 11:2). What is particularly interestingabout this is that uncomplicated CD is twice as prevalent among females as among males.[27] A possible

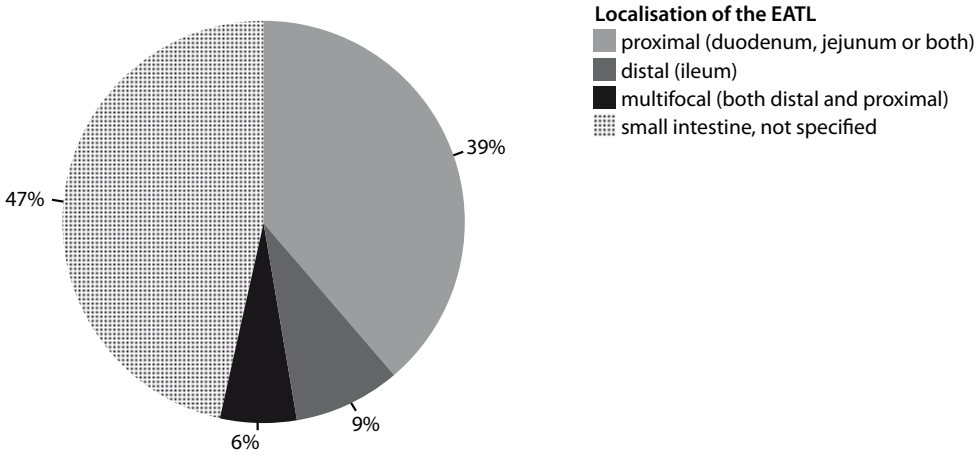

Figure 3. Localization of the histological specimen with enteropathy-associated T-cell lymphoma (EATL).

Marsh Classification	Total	No.%
Marsh classification specified	35	30%
Marsh 0	0 / 35	
Marsh I	2 / 35	
Marsh II	1 / 35	
Marsh IIIA	3 / 35	
Marsh IIIB	17 / 35	
Marsh IIIC	12 / 35	
CD not specified	20	17%
CD not mentioned, only 'EATL'	61	53%
Total	116	100

Table 2. The Marsh classification in the registry data.

explanation might be that more males remain undiagnosed and CD is only identified once a lymphoma has developed. Timely diagnosis of CD is important, since adherence to a GFD is known to play a protective role in preventing or reducing the risk of lymphoma development in CD.[17] In a study on CD in The Netherlands, males were indeed found to have unrecognised CD more often than females, whereas CD again was twice as prevalent in females.[28] Further studies will have to provide insight into whether and why men could be more prone to developing EATL. Possibly, there are additional independent genetic factors at play here, which may be gender related. We have reported previously that HLA-DQ2 homozygosity and the *MYO9B* gene are independent genetic risk factors for developing complicated CD.[29;30]

Most EATL's in our study were localized in the proximal small intestine, particularly the jejunum. This is consistent with reports elsewhere[23;31] showing that EATL commonly develops in this site but may also be found in the ileum and lymph nodes and less frequently in the stomach and colon. EATL is often multifocal with ulcerative lesions, which explains the high perforation rate at presentation or during chemotherapy.[31] The diagnosis was mostly made by surgical resection. On the one hand, surgery was necessary to establish the diagnosis, but it also constituted part of the treatment, as reported in other studies.[23;31] Only in a minority of cases the diagnosis could be established solely based on endoscopic biopsies, possibly due to the localization as well as the clinical presentation. In our own experience, since double balloon enteroscopy (DBE) [32] is available, with the possibility of taking diagnostic biopsies of the entire small intestine, surgery is nowadays mainly part of the treatment. With the arrival of these new small-bowel endoscopy techniques (VCE and DBE) and the developments in small-bowel radiological imaging (PET scan, MRI-enteroclysis and CT-enteroclysis) [33;34], earlier detection of malignant and premalignant lesions may be improved, leading, it is hoped, to a progress in the standard of care of these patients with a currently dismal outcome.

A search of a population-based registry rather than relying on reports of tertiary referral centres has major advantages, by giving a more reliable overview of the overall incidence

and excluding referral biases. A disadvantage, given the rarity of this disease entity, is that low numbers of cases can affect the overall incidence. Therefore it is particularly important whether and how the histopathological data are reported. In this search, 52 intestinal lymphomas were encoded as "malignant lymphoma", without specification of a B- or T-cell type. Given the distribution of the subtypes of the specified intestinal NHL's, it could be expected that up to 7 of these malignant lymphomas might be EATL and this would already affect the overall incidence of this disease entity. There might also be some degree of underreporting of EATL cases in *PALGA*, owing to the fact that the tumor is not recognized and patients just "fade away". Some patients present with non-specific worsening symptoms of diarrhoea and malabsorption in or after the 6th decade, and imaging techniques do not recognise abnormalities in a subgroup at this stage.[2;31] If no surgery for obstruction or perforation takes place, these patients might die of malnutrition or cachexia and will not be registered in the PALGA database unless autopsy is performed.
Furthermore, the histopathological reports registered in *PALGA* were concise; full specification of the exact localization of the EATL in the small intestine (47% not specified in Figure 3) and the Marsh criteria (in 70% of cases, Table 2) were often missing in this study. There was also a paucity of clinical information in these reports; consequently no data on the time of diagnosis of CD, the adherence and compliance to the GFD, and the subsequent histological and clinical response could be reported. Other studies have already elucidated that adherence to a GFD for more than 5 years has a protective effect and will reduce the overall cancer risk in CD to that of the general population.[1;17]
Adult CD is greatly underdiagnosed in The Netherlands as compared to other European countries. A study in 50,760 individuals representative of the adult Dutch population showed a prevalence of recognized CD of 0.016% (one of the lowest in Europe) and of unrecognized CD of 0.35% (comparable to that in other European countries).[28] Given the abovementioned protective effect of the GFD, it is tempting to speculate that this underdiagnosis may have affected the incidence of EATL found in the present study. Future studies on the incidence figures of EATL in other European populations may help to elucidate whether similar incidence figures of EATL can be expected.
Taking the possibility of underreporting into consideration, we conclude that EATL is a rare disease with an incidence of 0.10 per 100.000 inhabitants per year, that is more prevalent in males and has a peak incidence in the 7th decade of life. Although rare, EATL is a serious illness with a high mortality rate. Therefore, we believe that particularly patients over 50 years of age with newly diagnosed CD who develop worsening of symptoms of diarrhoea and malabsorption should be considered for complete endoscopic and radiologic evaluation to exclude EATL.

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4.
Flow cytometric determination of aberrant intra-epithelial lymphocytes predicts T-cell lymphoma development more accurately than T-cell clonality analysis in Refractory Coeliac Disease.

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Abstract

Background: Refractory Coeliac Disease (RCD) patients with aberrant, often clonal, intraepithelial T-cells are at high risk for development of enteropathy associated T-cell lymphoma (EATL). Early detection of those patients that actually develop EATL is of utmost importance for curative intervention.
Aim: First, to establish an optimal cut-off value for the percentage of aberrant lymphocytes, previously determined based on clinical observations, via reference ranges for aberrant T-cells in the duodenal mucosa of coeliac disease patient and control groups. Secondly, to compare aberrancy with intestinal T-cell clonality, as a prognostic parameter for EATL development in RCD.
*Methods: I*mmunophenotyping using flow cytometry was performed on small intestinal biopsy-derived lymphocytes, obtained from distinct coeliac disease (CD) patient and control groups (N=167 in total). T-cell clonality in duodenal biopsy specimens was assessed by PCR in RCD, ulcerative jejunitis and EATL patients (N=31 in total).
Results: In 95% of non-refractory CD patients, the highest percentage aberrant T-cells was 20%. Using this cut-off value, EATL development was exclusively seen in RCD with more than 20% aberrant T-cells (median 52% aberrant T-cells, range 27-94%). When compared with T-cell clonality analysis, >20% aberrancy showed a much higher negative predictive value and sensitivity (both 100%) for EATL development in RCD patients than T-cell clonality analysis (respectively 75% and 78%).
Conclusions: Quantification of aberrant T-cells by flow cytometry is preferable to T-cell clonality analysis for identification of RCD patients at risk for EATL development. A cut-off value of 20% is of use in risk stratification, therapeutic options and subsequent follow-up of RCD patients.

Introduction

Patients with CD are defined as suffering from refractory coeliac disease (RCD) when clinical and histological symptoms persist or recur, after a former good response to a strict gluten free diet (GFD), despite strict adherence to the diet for more than 12 months, unless earlier intervention is necessary.[1-4] RCD patients are nearly always adults of 50 years and over. A high percentage (52%) of these patients develops an enteropathy associated T-cell lymphoma (EATL) within 4-6 years, which is the main cause of death in this patient group and has a 5-year survival of only 8%.[5] Early identification of these patients allows for early therapeutic intervention with a significant reduction in morbidity and mortality.[6]However, reliable identification of these patients remains difficult.
To identify patients at risk for EATL development, the presence of a detectable monoclonal T-cell population in intestinal biopsies has been associated with an increased risk for EATL development. [2;7-10] However, positive and negative predictive value of this method are disputed.
The presence of aberrant intraepithelial lymphocytes (IELs) appears to be a more reliable prognostic marker. According to the guidelines of the European Coeliac Disease

working group[11] RCD patients are subdivided into RCD type I and type II patients, with phenotypically normal and aberrant IELs, respectively. IELs are considered aberrant when expressing cytoplasmic CD3ε, but lacking surface expression of T-cell markers CD3, CD4 and CD8. [7;12] The presence of these IELs is associated with a significant increase in EATL development.[2;5;13;14]

Considering the fact that RCD II patients are at a high risk for development of EATL, accurate discrimination between both types of RCD is of utmost importance.[5] However, the diagnostic criteria of RCD I and II, based on aberrant IELs, have not been clearly defined. Large series of patients are missing and no cut-off values have been determined so far based on laboratory data. In literature, using immunohistochemistry, the percentage of CD3+ cells without CD8 expression among IELs was statistically higher in RCD (n=10, 48-98%) than in patients with CD (n=10, 2-33%) and controls (n=5, 0-42%).[12] However, immunohistochemical analysis cannot distinguish between intracellular and surface expression of CD3, which is important since we have also observed surface CD3-CD8+ 'atypical' aberrant T-cells in an RCD patient who eventually evolved to a CD8+ small cell EATL (unpublished data). Moreover, the presence of significant numbers of intraepithelial CD4 cells may hamper proper interpretation of the CD3/8 ratio.

Multiparameter flow cytometry is more specific and sensitive when compared with single-parameter immunohistochemical analysis and can be used for adequate quantification. Furthermore, the use of flow cytometry allows for discrimination between intracellular and surface antigen expression. This technique does, however, ask for a well validated cut-off value. Thus far, no reference ranges for intestinal IEL subsets have been reported for distinguishing RCD I from RCD II patients.

Therefore in the present paper we set out to validate an optimal cut-off value for the percentage of aberrant IELs in RCD, previously determined based on clinical observations,[5] via reference ranges for aberrant T-cells in the duodenal mucosa in different CD patient and control groups. Subsequently, we compared the predictive value of percentages of aberrant intra-epithelial T-cells with intestinal T-cell clonality analysis, for EATL development in RCD patients.

Patients, material and methods

Patients

Flow cytometric analyses on 3-4 small intestinal spike biopsy specimens was performed in 167 consecutive subjects evaluated for RCD and CD between June 2003 and August 2006. To avoid bias only biopsies taken before therapy were included in this study. The included patients were subdivided into 5 subgroups:

I Controls without CD, (n=49, 13M/36F, mean age 41 years, SD 14 years, range 41-63 years). This group consisted of patients who had small intestinal biopsy specimens for exclusion of CD, symptoms varied from aphtous stomatitis, reflux, nausea and dyspepsia to diarrhoea, weight loss, abdominal pain and osteopenia. Some patients had a family member with CD and were evaluated to exclude CD as a potential cause of fatigue or non-specific abdominal complaints. None of these controls were on a GFD. All patients had normal histology and negative antibody tests.

II Untreated CD (n=17, 5M/12F, mean age 45 years, SD 19 years, range 17-71 years). All patients had symptoms of malabsorption, antibodies against both endomysium (EMA) and tissue transglutaminase (tTGA) and villous atrophy on histological examination. These patients were not on a GFD yet.

III Treated CD patients (n=60, 21M/39F, mean age 51 years, SD 16 years, range 14-76 years) responding on a GFD (on average 55 months on a GFD (range 3-396 months, SD 81). The diagnosis of CD was confirmed by histological examination with a documented histological response to gluten withdrawal [3].

IV Patients with RCD, considered to be refractory when symptoms of malabsorption due to villous atrophy persisted or recurred after a former good response on a GFD. Histopathology of these patients showed at least partial villous atrophy (Marsh IIIA) and other causes of villous atrophy had been excluded.[3;4] Taking in consideration that a significant number of patients (around 50%) suspected for RCD may indeed have inadvertent gluten ingestion[15] the dietary compliance was checked by a dietician, and confirmed by negative serology for tTGA antibodies. In the RCD patients, the presence of EATL has been excluded using radiological and endoscopic methods (small intestinal follow through, computed tomography scanning of thorax and abdomen,[16] whole body positron emission tomography scan,[17] upper gastrointestinal endoscopy, video capsule endoscopy and/or double balloon enteroscopy,[18] as well as trephine bone marrow biopsy specimens). The techniques for the video capsule endoscopy and double balloon enteroscopy are available in our centre since the beginning of 2003, after which the study started. In all patients at least 3 of these techniques were used in the extensive work-up to exclude EATL. The majority of the RCD patients included in this study has also been described in our recent study on survival in refractory coeliac disease.[5]

   RCD I (n=16 , 3M/13F, mean age 54 years, SD 10 years, range 35-70 years) and RCD II (n=17, 7M/10F, mean age 65 years, SD 8 years, range 51-88 years).

V Patients inititally presenting with an EATL and/or ulcerative jejunitis (n=8 of whom 2 UJ, 6M/2F, mean age 60 years, SD 8 years, range 48-73 years) often due to perforation or bowel obstruction. In primary EATL, there is no history of complicated CD and none of them have followed a GFD. The diagnosis of EATL was established according to the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues.[19;20]

   The immunohistochemical features of EATL are the presence of large or medium size T-cell proliferation and can be CD3+ CD8- CD30+ large cell lymphomas or CD3+ CD8+ CD30- small cell lymphomas. Diagnosis of EATL was confirmed by an expert- panel of pathologists.

Small intestinal biopsy specimens

During upper endoscopy large spike forceps biopsy specimens (Medi-Globe®) were taken from the second part of the duodenum.[3] Four to six biopsy specimens were fixed and preserved in 10% formalin for histopathological evaluation. In patients referred for suspected RCD, 2 biopsy specimens were taken for TCR gene rearrangement

studies and preserved on histocon, frozen at –20°C. For flow cytometric evaluation 3-4 biopsy specimens were taken and immediately analyzed. All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.

Isolation of intestinal lymphocytes and flow cytometry

Intestinal lymphocytes were isolated from 3 to 4 duodenal biopsy specimens by homogenizing the biopsy specimens directly, without chemical or enzymatic treatment, through a 100µm, followed by a 40µm nylon cell strainer (BD Biosciences® (Discovery Labware), Bedford MA) in PBS medium supplemented with 1% FCS. The released cells were subsequently washed and stained for 30 min. on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC) conjugated monoclonal antibodies directed against CD3, CD4, CD8, CD7, CD45, γδTCR, CD19, and CD16/56 (all from from BD Biosciences, San Jose CA) and CD103 (IQ products, Groningen The Netherlands). Cytoplasmic CD3 staining was performed after cell permeabilization (Cytofix/CytoPerm Plus™ kit by BD). Four color antibody-combinations included the following (FITC/PE/PerCP/APC): CD3/CD8/CD45/CD4, CD3/CD16+56/CD45/CD19, IgG1/IgG1/CD45/IgG1, CD103/CD7/CD45/CD3, x/TCRγδ/CD45/x, cytoplasmic IgG1/CD7/CD45/CD3 and cytoplasmic CD3/CD7/CD45/CD3. The antibodies used in this study are listed in **table 1**.

Nr.	Reagens	Company	mAb clone
1	Multitest CD3-FITC CD8-PE CD45-PERCP CD4-APC	BD	CD3 (SK7) CD8 (BK1) CD45 (2D1) CD4 (SK3)
2	Multitest CD3-FITC CD16+56-PE CD45-PERCP CD19-APC	BD	CD3 (SK7) CD16 (B73.1), CD56 (NCAM16.2) CD45 (2D1) CD19 (SJ25C1)
3	CD3-FITC	BD	Clone SK7
4	CD3-APC	BD	Clone SK7
5	IgG1-FITC	BD	Clone X40
6	IgG1-PE	BD	Clone X40
7	IgG1-APC	BD	Clone X40
8	CD103-FITC	IQ products	Clone B-ly7
9	CD7-PE	BD	Clone M-T701
10	TCR-γδ-PE	BD	Clone 11F2
11	CD45-PERCP	BD	Clone 2D1

Table 1. Fluorochrome-labelled antibodies used for flow cytometry.

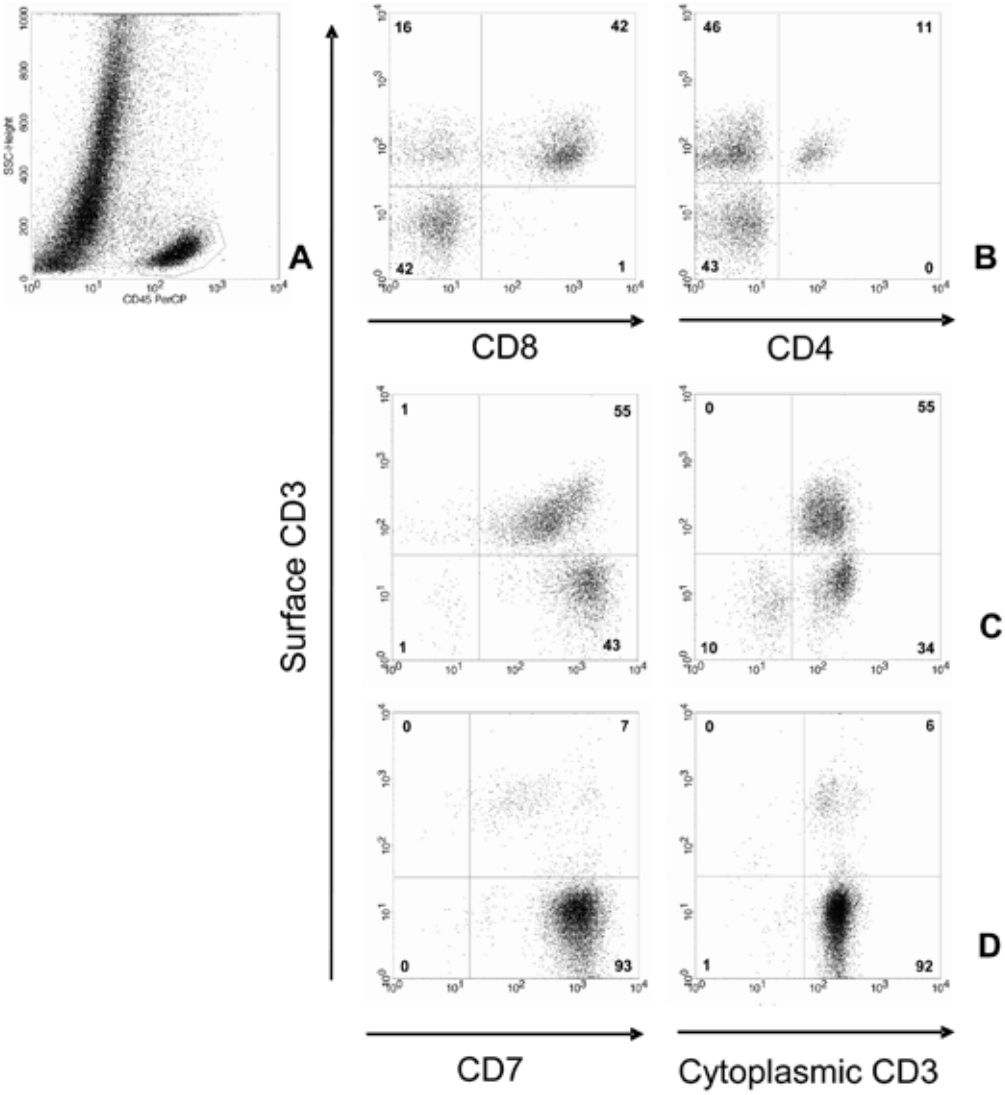

Figure 1 Example of flow cytometric analyses of intestinal lymphocytes isolated from duodenal biopsy specimens. (**A**) Lymphocyte selection gate based on CD45 positivity and low sideward scatter. (**B**) Shows a population surface CD3 negative lymphocytes that do not express CD8 nor CD4. (**C**) The aberrant T-cell population in this patient, 34–43%, left: double staining of surface CD3–CD7+ cells shown within the CD103+/CD45+ cells; right: surface CD3–, cytoplasmic CD3+ cells, double staining shown within the CD7+/CD45+ cells. (**D**) As a comparison, the aberrant T-cell population in another patient with almost exclusively aberrant T-cells in the duodenal mucosa (92–93%).

Flow cytometric analysis was performed on a standard 4-color Fluorescence Activated Cell Scanner (FACSCalibur, BD Biosciences, San Jose CA). The data were analyzed using the Cellquest Pro software program (Becton Dickinson). Care was taken to analyze only viable cellular events based on light scatter properties. All analyses were performed on lymphocytes, based on bright CD45 staining and low sideward scatter. Isotype-matched control antibodies were used in all cases to assess background fluorescence. Intraepithelial localization of lymphocytes was confirmed by surface expression of CD103, a member of the integrin family (αEβ7).[21] Aberrant T cells were defined as surface CD3- $CD7^+$ cells (**Figure 1C&D** left dot plots, double staining shown within the CD103+/CD45+ cells) and as surface CD3- cytoplasmic $CD3^+$ cells (**Figure 1C&D** right dot plots, double staining shown within the CD7+/CD45+cells). In the latter analysis the cytoplasmic CD3 expression of the aberrant T-cells has to be at least as strong as the normal surface CD3+ T-cell population, to gate out NK-cells.[22] In case there is a discrepancy between these two values, we regard the latter value as most important, as cytoplasmic CD3 expression represents the only lineage-defining T-cell marker, in contrast to CD103, which is also important for the intraepithelial localisation, but less specific and can, for instance, also be found in hairy cell leukemia on B-cells and intestinal NK IELs.[22;23]

Histopathology

Histopathological findings were classified using the modified Marsh criteria for the gluten sensitive spectrum.[11;24-26]

Assessment of T-cell clonality

T-cell receptor-gamma (TCR-γ) gene rearrangements were analyzed on entire cryopreserved biopsy specimens. DNA was extracted from cryosections by a standard procedure using proteinase-K digestion and ethanol precipitation of the genomic DNA. TCR-γ gene rearrangements were subsequently analyzed by multiplex polymerase chain reaction (PCR) amplification, using the primers and probes provided by the BIOMED-2 consortium according to their guidelines.[27]

Statistical analysis

One way ANOVA analysis of the data, for comparison between the groups, was performed using SSPS software (version 11.0, SPSS Inc., Chicago, Illinois). To correct for multiple testing, post hoc pair wise comparisons using Tukey's honestly significant difference test was carried out. A value of $p<0.05$ was considered statistically significant.

Results:

A cut-off value of 20% aberrant T-cells in RCD

In an initial pilot study[28] first degree relatives of CD patients did not differ from the control group without CD with respect to IEL subsets. Therefore in the present study first degree relatives were combined with the other non-CD controls. **Figure 1** depicts an example of flow cytometric analyses of IELs isolated from duodenal biopsy specimens and reference ranges for all intestinal lymphocyte subsets are displayed in **table 2**.

Subset	Controls Without Coeliac Disease N=49	Untreated Coeliac Disease N=17	Coeliac Disease on GFD N=60	RCD I N=16	RCD II N=17	Primary EATL and/or UJ N=8a
CD3+ T-cells						
Median	86	94	90	93	43	90
10^{th}- 90^{th} percentile	78-93	80-97	78-97	81-98	16-63	55-94[a]
CD4+ T-cells						
Median	24	19	18	13	13	19
10^{th}- 90^{th} percentile	10-44	9-32	7-38	5-29	3-17	11-21[a]
CD8+ T-cells						
Median	56	67	61	70	20 [b]	63
10^{th}- 90^{th} percentile	39-76	41-81	42-79	52-88	2-31	25-64[a]
CD7+ lymphocytes						
Median	96	95	96	95	96	94
10^{th}- 90^{th} percentile	88-98	85-99	88-98	91-99	90-98	58-96[a]
TCR γδ+ T-cells						
Median	9	11	13 [c]	17	5 [d]	13
10^{th}- 90^{th} percentile	1-18	5-35	5-28	6-23	1-14	4-28[a]
CD19+ B-cells						
Median	0.5	2	1	1	1	2
10^{th}- 90^{th} percentile	0.1-3	0.4-12	0.1-6	0.01-3	0.2-8	0.01-13[a]
CD16/56+ NK-cells						
Median	7	3	5	3	5	4
10^{th}- 90^{th} percentile	3-14	1-7	1-12	1-10	1-17	0.4-5[a]
CD103+ IELs						
Median	82	83	84	84	84	72
10^{th}- 90^{th} percentile	67-95	63-90	69-94	36-95	73-93	44-80[a]
CD7+ CD3- aberrant T-cells within CD103+ cells						
Median	8	2	5	3	37 [e]	4
10^{th}- 90^{th} percentile	4-16	0.6-11	1-16	0.5-18	28-74	1-8[a]
CD7+ CD3- cytCD3+ aberrant T-cells						
Median	4	1	2	2	52 [f]	2
10^{th}- 90^{th} percentile	1-9	0.07-4	0-5	0.5-10	34-89	0.4-7[a]

Table 2 Lymphocyte subsets in duodenal biopsy specimens as % of intestinal lymphocytes, determined by Flow cytometry

[a] In the primary EATL de novo group, the 75th percentile is provided; due to the small group, it was not possible to give the 90th percentile.

[b] Significantly less CD8+ T-cells in RCD II as compared to all other groups, in all cases p<0.0001.

[c] Significantly more TCRγδ+ lymphocytes in CD on a GFD as compared to controls without CD, p=0.001.

[d] Significantly less TCRγδ+ lymphocytes in RCD II as compared to all other groups, with exception of the controls; CD on GFD, pb0.0001; active CD, p=0.013; RCD I, p=0.042; EATL de novo, p=0.043.

[e] Significantly more aberrant T-cells in RCD II as compared to all other groups, in all cases p<0.0001.

[f] Significantly more aberrant T-cells in RCD II as compared to all other groups, in all cases p<0.0001.

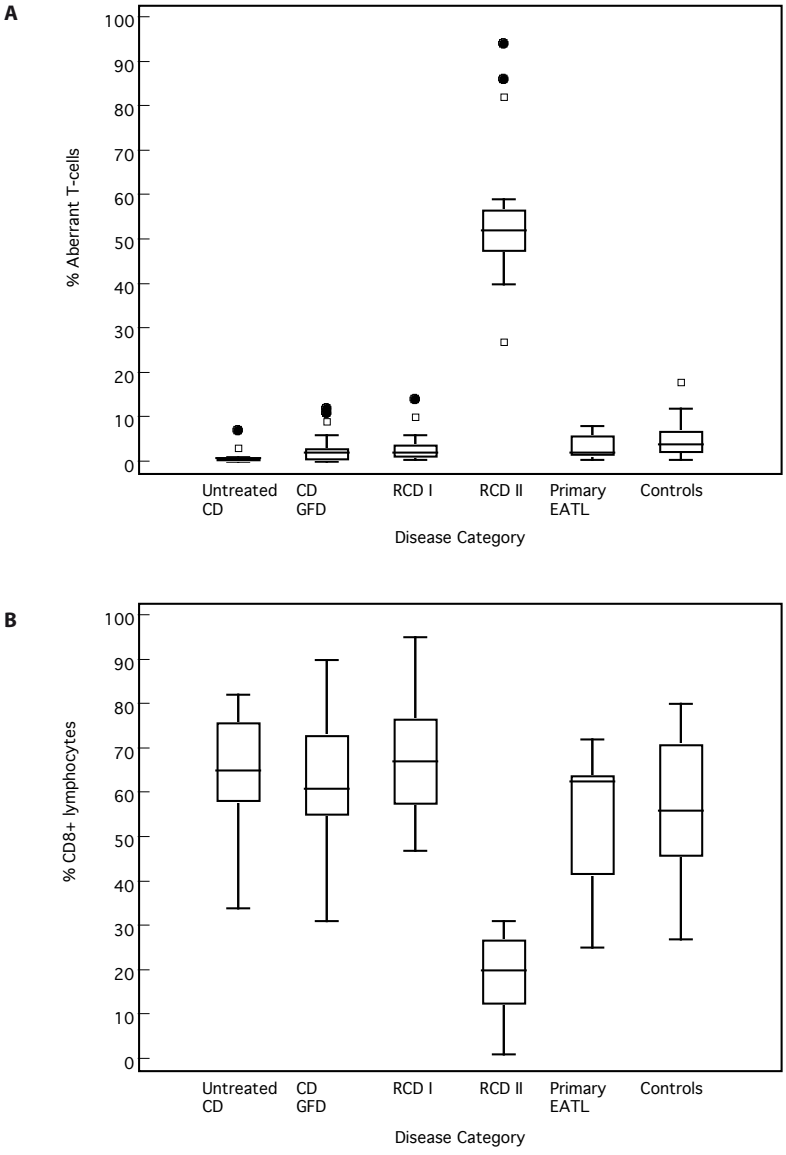

Figure 2 (**A**) Percentage aberrant T-cells (CD7+ surface CD3– cytoplasmic CD3+) in duodenal biopsy specimens of each disease category. There were significantly more aberrant T-cells in the RCD II group as compared to all other groups, in all cases p<0.0001. 95% confidence intervals: untreated CD (CI: 44–62), CD on a GFD (CI: 45–59), RCD I (CI: 41–59), controls without CD (CI: 41–56), EATL (CI: 40–61). (**B**) Percentage CD8+ lymphocytes in duodenal biopsy specimens of each disease category. There were significantly less CD8+ T-cells in RCD II as compared to all other groups, in all cases p<0.0001. 95% confidence intervals: untreated CD (CI: –60–33), CD on GFD (CI: –55–32), RCD I (CI: –63–35), controls without CD (CI: –49–26), primary EATL and/or UJ (CI: –53–18).

	RCD evolving to EATL n=10	RCD without EATL n=13
Detection of aberrant IELs [1]		
> 20% aberrant IELs	10	7
< 20% aberrant IELs	0	6
T-cell clonality analysis [2]		
Monoclonal	7 *	7
Polyclonal	2	6

Table 3. Comparing the detection of percentages aberrant T-cells with T-cell clonality analysis.

[1] Sensitivity and specificity of this test in RCD patients with regard to EATL development are 100% and 46% resp. The negative predictive value is 100%. The positive predictive value is 59%.

[2] Sensitivity and specificity of this test are 78% and 46% resp. The negative predictive value is 75%. The positive predictive value is 50%.

* Clonality analysis was inconclusive in 1/10 RCD patients with EATL due to poor quality of the DNA.

In 95% of non-refractory CD and control patients, the highest percentage aberrant T-cells in duodenal biopsy specimens was 20%. Therefore, a cut-off of 20% was used in the present study for discrimination of RCD I and II patients. The percentage of aberrant T-cells in duodenal biopsy specimens of each disease category is shown in **figure 2A**. Distribution of aberrant T-cells throughout CD patient and control groups shows medians of 1-8% in all these groups versus 52% in RCD II (range 27-94%, p<0.0001, **table 2**). In agreement with this, the percentage of CD8 positive lymphocytes was significantly lower in RCD II as compared to all other groups (p<0.0001 in all cases, **figure 2B**), since the normal CD8 expression is lost upon progression to an aberrant phenotype.

Flow cytometric analyses and EATL development

The predictive value of the presence of >20% aberrant T-cells with regard to EATL development in RCD patients, as compared to clonality, is depicted in **table 3**.

All the RCD patients who evolved to EATL (n=10, mean 20 months after the diagnosis RCD, SD 13, range 7-43) had >20% aberrant T-cells. This indicates a high sensitivity of 100%. None of the RCD patients with <20% aberrant T-cells developed EATL (within mean 44 months follow up, SD 19, range 21-100), thus the NPV is 100%. The PPV for EATL development in case of >20% aberrant T-cells is 59%. Interestingly, in this study the aberrant T-cells in duodenal biopsy specimens of primary EATL and UJ patients were below 20% (**figure 2, table 2**). Here, cells with an aberrant phenotype, which are often clonal, appear to be largely confined to the tumor mass and/or ulcerations and cannot be found diffusely spread throughout the small intestine as in RCD II.

T-cell clonality and EATL development

Previous studies have shown that a clonal T-cell population can be found in the intestinal mucosa of RCD patients. It was suggested that EATL development is closely correlated with the presence of these clonal T-cells. [2;8;29] To investigate this in the current

RCD group, we analyzed TCRγ gene rearrangements in duodenal biopsies of 17 RCD II patients (of whom 10 developed large cell EATL), 6 primary EATL, 2 UJ and 6 RCD I patients just below the 20% cut-off.
The results of this analysis are depicted in **table 3**. The sensitivity of this test for the development of EATL in RCD was 78%, as not all RCD patients who evolved to EATL had a monoclonal T-cell population in the duodenal biopsy specimen (NPV 75%). Of the RCD patients with a monoclonal population half developed EATL, indicating a PPV of 50%. TCR gene arrangement analysis was performed in entire duodenal biopsies of all RCD patients.
Detected clonal rearrangements could be compared with the clonal TCR rearrangement of the EATL only 2/7 monoclonal RCD patients evolving to EATL. In these cases the clone detected in the EATL corresponded to the clone detected in the duodenal biopsy of the patient.
The 6 primary EATL patients all had a clonal TCRγ gene rearrangement in the tumour, but only 2/6 had a clonal T-cell population in the duodenal biopsy specimen at that time, identical to the tumor. A clonal population was present in the non-ulcerated duodenal biopsy specimen of 1/2 UJ patients. No TCRγ gene rearrangement was assessed in the ulcerated areas.

Discussion

In this study, we set out to validate an optimal cut-off value for the percentage of aberrant IELs in RCD via reference ranges for aberrant T-cells in the duodenal mucosa in CD patient and control groups, using flow cytometry of intestinal lymphocytes. In 95% of non-refractory CD and control patients the highest percentage of aberrant T-cells in duodenal biopsy specimens was 20%. This is in agreement with the cut-off, which had been previously suggested in the RCD group based on the clinical observation that none of the RCD patients with less than 20% aberrant T cells eventually developed EATL.[5] In contrast, 52% of the RCD II patients described in our recent study on survival in RCD developed EATL within 4-6 years after the diagnosis of RCD II, with a 2 year survival rate of only 15-20%.[5] Therefore a definite cut-off of 20% was used for discrimination of RCD I and II. Distribution of aberrant T-cells throughout CD patient and control groups shows medians of 1-8% in these groups versus 52% (range 27-94%) in RCD II ($p<0.0001$). In agreement with this, the percentage of CD8 positive lymphocytes was significantly lower in RCD II as compared to all other groups ($p<0.0001$ in all cases), since the normal CD8 expression is lost upon progression to an aberrant phenotype.
In the guidelines of the CD working group, UEGW Amsterdam 2001, patients with ulcerative jejunitis are considered to be RCD II patients, since their histology is compatible with CD and needs intervention, but is not yet compatible with small intestinal lymphoma.[11] In the present study, the aberrant T-cells in UJ patients (and primary EATL) appear to be largely confined to the tumor mass and/or ulcerations and have not been found diffusely spread throughout the small intestine as is the case in RCD II and secondary EATL patients.[5] This may reflect a different pathogenesis with respect to UJ and primary EATL development. We currently hypothesize that these UJ patients may be

considered as a separate entity from RCD II, probably a low-grade EATL. However, as this hypothesis is based only on a few patients, further studies are required.
Regarding T-cell clonality, we compared the established cut-off value of 20% aberrant T-cells to this frequently used pre-malignant parameter, for the predictive value of EATL development in a group of RCD patients. Daum et al [29] have shown that a clonal γ-gene rearrangement could be found in the duodenal biopsy specimen of 3/8 patients with a resected EATL, 2/2 with UJ, 2/3 with RCD evolving to EATL and in 1/6 RCD, whereas clonal TCR-γ gene rearrangements were present in all EATL specimens. Similar results were found in the present study. Statistical analysis of our data revealed a much higher negative predictive value and sensitivity (both 100%) for aberrant T-cells with regard to EATL development in RCD, when compared to clonality in a duodenal biopsy specimen (75% and 78% respectively). The positive predicitive values of these tests for EATL development in RCD are almost comparable. The majority of the RCD patients with phenotypically aberrant IELs has a monoclonal T-cell population, as shown in this study as well as other studies. [2;7;8]
Immunohistochemical analysis of duodenal biopsies has an important role in the diagnosis and risk stratification of CD, especially in patients diagnosed over 50 years of age. For the distinction between RCD I and II, however, in our opinion immunohistochemistry is neither sufficiently sensitive nor specific, due to the fact that immunohistochemical analysis can't distinguish between intracellular and surface expression of CD3. Since intracellular CD3 expression without surface CD3 expression represents a hallmark of aberrant IEL, discrimination of both types of CD3 expression is of utmost importance. Especially, since we have also observed surface CD3- CD8+ 'atypical' aberrant T-cells in a RCD patient who eventually evolved to a small cell EATL (unpublished data). Moreover, in case of a significant amount of CD4 cells, which are also present in the small intestinal mucosa, the CD3/8 ratio on immunohistochemistry can already be disrupted, which is not caused by aberrant T-cells.
In conclusion, quantification of aberrant T-cells by flow cytometry is well suited for the identification of those RCD patients at risk for EATL and has a higher predictive value and sensivity than T-cell clonality analysis of duodenal biopsy specimens. A cut-off value of 20% appears reliable for early risk stratification [5] and targeted therapeutic options in RCD patients.[6;30;31], representing the projected benefits of the described diagnostic differentiation. This is particularly important since once overt lymphoma has developed, treatment outcome and survival are very poor.[5;32] Additionally, quantification of aberrant T-cells is useful for the subsequent follow-up of treated RCD II patients.[6]

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Part three
Pathogenesis

5.
Aberrant T-lymphocytes in Refractory Coeliac Disease are not strictly confined to a small intestinal intraepithelial localization.

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Abstract

Background: Refractory Coeliac Disease (RCD) is characterized by persisting mucosal pathology in spite of a strict gluten free diet (GFD). In RCD type II phenotypically aberrant (CD7+CD3-CD4/8-cytoplasmicCD3+) T-lymphocytes are present within the Intraepitelial Lymphocyte (IEL) population in the small intestine, and 50-60% of these patients develops an Enteropathy Associated T-cell lymphoma (EATL).
Aim: To investigate whether aberrant T-lymphocytes in RCD II can be detected in other parts of the small intestinal mucosa besides the intraepithelial compartment. Additionally, the presence of aberrant T-lymphocytes was analyzed in two RCD II patients that developed atypical skin lesions.
Methods: Multiparameter flow cytometric immunophenotyping was performed on both IEL and lamina propria lymphocyte (LPL) cell suspensions, isolated from small bowel biopsy specimens of RCD II patients (n=14), and on cutaneous lymphocytes isolated from skin-lesion biopsy specimens of RCD II patients (n=2). In addition, immunofluorescence analysis of frozen RCD II derived small intestinal biopsies was performed.
Results: Our results clearly show that aberrant T-lymphocytes may be present in both the IEL and the LPL compartments of RCD II derived small intestinal biopsies. Although the highest percentages are always present in the IEL compartment, aberrant LPL can exceed 20% of total LPL in half the RCD II patients. Interestingly, cutaneous lymphocytes isolated from atypical skin lesions that developed in some RCD II patients showed a similar aberrant immunophenotype as found in the intestinal mucosa.
Conclusions: In RCD II the aberrant T-lymphocytes may also reside in the subepithelial layer of the small intestinal mucosa, in the lamina propria, and even in extraintestinal localizations including the skin. Whether this phenomenon represents a passive overflow from the intestinal epithelium or active trafficing towards other anatomical localizations remains to be elucidated. RCD II appears to be a disseminated disease, which may impose the risk of EATL development outside the intestine.

Introduction

Coeliac disease (CD) is a T-cell mediated disease of the small intestine triggered by the ingestion of dietary wheat gluten in genetically predisposed individuals.[1,2] It commits the patients to a life-long glutenfree diet, which is sufficient to treat the overwhelming majority of patients. However, a small group of these patients, mainly those diagnosed above the age of 50, fails to improve histologically and clinically upon elimination of gluten from the diet. These patients are regarded as suffering from Refractory Coeliac Disease (RCD).
According to the guidelines of the European Coeliac Disease working group[3] RCD patients can be subdivided into RCD type I and type II patients, with phenotypically normal and aberrant intraepithelial T-lymphocytes (IEL) in the small intestinal mucosa, respectively. IELs with an aberrant immunophenotype are known to be a prognostic parameter in RCD since their presence is associated with the development of EATL. Cellier et al. have first shown that RCD is associated with this abnormal subset of IELs of

T-cell origin, expressing cytoplasmic CD3ε and restricted rearrangements of the TCRγ chain, but lacking surface expression of T-cell markers CD3, CD4 and CD8.[4] When normal expression of T-cell surface markers occurs (RCD I), the prognosis is less dismal than when an aberrant intraepithelial lymphocyte population is present (RCD II), 50-60% of the latter patients develops EATL within 4-6 years, which has a very poor prognosis and a 5-year survival of only 8%.[5] These EATLs are thought to arise from the IEL compartment, and share immunophenotypic characteristics with to the aberrant IELs in RCD II.[6;7] Since the presence of aberrant IELs is directly associated with a significant risk of EATL development[5;8-10], the therapeutic challenge in these RCD II patients is to identify and subsequently target the aberrant IELs to eventually prevent EATL development.

Considering the fact that RCD II patients are at a high risk for development of EATL, accurate discrimination between both types of RCD is of utmost importance.[5] In our previous work we showed that quantification of aberrant IELs by flow cytometry is well suited for the specific identification of RCD II patients.[11] This was recently confirmed by another group, that also demonstrated flow cytometric immunophenotyping of intestinal IEL to be a useful tool in the diagnostic work-up of patients with RCD.[12]

With regard to the potential of aberrant T-cells to disseminate, an immunohistochemical study by Verkarre et al.[13] showed diffuse intraepithelial spreading to different longitudinal levels throughout the intestinal tract. The presence of aberrant T-cells was found to extend to the gastric as well as the colonic mucosa. Previous studies by Cellier et al. [4;8] already detected the aberrant T-cell population in the blood and colon of four RCD II patients. No studies on the cross-sectional levels of aberrant T-cells in the small intestine have been performed sofar. This may be interesting as EATL are known to have the ability to spread to an extraintestinal level,[14] and the Lamina propria compartment is in direct contact with the blood stream. Therefore, in the present study we set out to investigate whether aberrant T-lymphocytes in RCD II can be detected in other parts of the small intestinal mucosa besides the epithelial compartment. Additionally, to investigate potential spreading to an extraintestinal localization, the presence of aberrant T-lymphocytes was analyzed in two RCD II patients that developed multiple atypical skin lesions.

Patients, material and methods

Patients

Flow cytometric analyses on 3-4 small intestinal spike biopsy specimens were performed in 16 consecutive subjects evaluated for RCD between January 2006 and December 2007.

These patients with RCD II (n=16), were considered to be refractory when symptoms of malabsorption due to villous atrophy persisted or recurred after a former good response on a glutenfree diet (GFD). Signs and symptoms were comparable to those described in previous studies. [5] Histopathology of these patients showed at least partial villous atrophy (Marsh IIIA) and other causes of villous atrophy had been excluded, including Whipple's disease, Crohn's disease, tuberculosis, radiation enteritis, AIDS,

common variable immunodeficiency syndrome, eosinophilic gastroenteritis, autoimmune enteropathy and immunoproliferative small intestinal disease, giardiasis, postinfectious diarrhea, tropical sprue, collagenous sprue and protein intolerance.[15]
Considering the fact that a significant number of patients (around 50%) suspected for RCD may indeed experience inadvertent gluten ingestion, their dietary compliance was carefully evaluated by a dietician, and confirmed by negative CD serology.[16] The presence of an EATL was excluded at time of flow cytometric analysis by radiological and endoscopic methods, including small intestinal follow through, computed tomography scanning of thorax and abdomen,[17] whole body positron emission tomography scan,[18] upper gastrointestinal endoscopy, video capsule endoscopy and/or double balloon enteroscopy.[19]
Based on clinical presentation and flow cytometric analysis the RCD II patients were identified using the 20% cut-off value for aberrant IELs (surface CD3- CD4/8- CD7+ cytoplasmic CD3++), as established previously.[5;11] This cut-off value has proven to be reliable for early risk stratification [5] and targeted therapeutic options in RCD patients.[20-22] The total group consisted of 16 RCD II patients (7M/9F, mean age 62 years, range 46-72 years).

Isolation of IELs from small intestinal biopsy specimens and flow cytometry

During upper endoscopy large spike forceps biopsy specimens (Medi-Globe®) were taken from the second part of the duodenum.[23] For flow cytometric evaluation 3-4 biopsy specimens were taken and immediately analyzed. All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.
Multiparameter flow cytometric immunophenotyping was performed on both IEL and lamina propria lymphocyte (LPL) cell suspensions, isolated from small bowel biopsy specimens of RCD II patients (n=16), and on cutaneous lymphocytes isolated from biopsy specimens derived from atypical skin lesions of RCD II patients (n=2). In addition, immunofluorescence analysis of frozen RCD II derived intestinal biopsies was performed.
Intraepithelial lymphocytes were isolated from intestinal biopsies as originally described by Madrigal et al.[24] with minor modifications. Briefly, biopsies were vigorously shaken at 37˚C for 60 min in PBS supplemented with 1mM dithiothreitol (Fluka BioChemika, Buchs Switzerland) and 1mM Ethylenediaminetetraacetic (Merck, Darmstadt Germany). Lamina propria lymphocytes were obtained from the deepithelized mucosal tissue as described previously.[25] Briefly, following IEL isolation residual biopsy tissue was digested for 2-3 hours at 37°C by 128 U/ml collagenase (type 1A, Roche Diagnostics, Mannheim Germany). Subsequently, tissue was homogenized to release the LPL. Cutaneous lymphocytes were isolated from fresh skin biopsy specimens using a similar procedure as described above for LPL.
The released IELs and LPLs were washed twice with PBS supplemented with 0,1% BSA (Roche Diagnostics) and subsequently stained for 30 minutes on ice, with fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, CD7, CD45,

γδTCR (all from from BD Biosciences, San Jose CA). Cytoplasmic staining of CD3 was performed after cell permeabilization (Cytofix/CytoPerm Plus™ kit by BD Biosciences). Flow cytometric analysis was performed on a standard 4-color Fluorescence Activated Cell Scanner (FACSCalibur, BD Biosciences). The data were analyzed using Cellquest software (BD Biosciences). Care was taken to analyze only viable cellular events based on light scatter properties. All analyses were performed on lymphocytes, based on bright CD45 staining and low sideward scatter. Aberrant T cells were defined as CD7+ cytoplasmic $CD3^{++}$, surface CD3, CD4 and CD8 negative cells, as described previously.[11]

Histopathology and immunofluorescence analysis

Additional duodenal biopsy specimens taken at time of endoscopy were used for standard histopathological evaluation. Histopathological findings on sections of formaline-fixed biopsy specimens were classified using the modified Marsh criteria for the gluten sensitive spectrum.[26-28] Immunofluorescence analysis was performed on formalin-fixed paraffin sections using antibodies directed against human CD3, CD4 and CD8 (DAKO), using standard procedures. Specific staining was visualized by secondary FITC- (detecting CD3) and TRITC- (detecting CD4 and CD8) labelled antibodies (Southern Biotec, UK), and analyzed by standard confocal laser-scanning microscopy. Histopathological evaluation of skin biopsy specimens was performed analogously.
Assessment of T-cell clonality

T-cell receptor-gamma (TCR-γ) gene rearrangements were analyzed on entire cryopreserved biopsy specimens. DNA was extracted from cryosections by a standard procedure using proteinase-K digestion and ethanol precipitation of the genomic DNA. TCR-γ gene rearrangements were subsequently analyzed by multiplex polymerase chain reaction (PCR) amplification, using the primers and probes provided by the BIOMED-2 consortium according to their guidelines.[29]

Statistical analysis

To compare the proportions of aberrant T-cells between the epithelial and lamina propria compartments, linear regression and analysis of variance (ANOVA) was used. Analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, Illinois). A value of $p<0.05$ was considered statistically significant.

Results

A positive correlation between the presence of aberrant IELs and LPLs

Table 1 depicts the patient characteristics of all 16 RCD II patients included in the study. In all patients >20% aberrant IEL were detected. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were analyzed separately. A representative example of flow cytometric analysis of IEL and LPL within the one patient (no.1) is shown in **figure 1**. The presence of aberrant IEL and LPL was subsequently confirmed by immunofluorescence analysis in this same patient (**Figure 2**). Next to normal CD3+ CD4/8+ T-lymphocytes, the lamina propria clearly shows the presence of aberrant

Patientnumber	Sex & Age (M/F years)	Marsh at Diagnosis	HLA-DQ status	% aberrant IEL	Clonal TCR-γ rearrangement	Skin lesions
1	F66	IIIC	DQ2/2	93	Yes	Yes
2	M72	IIIA	DQ2/2	69	Yes	No
3	F59	IIIC	DQ2	69	Yes	No
4	F68	IIIC	DQ2	60	Yes	No
5	F63	IIIC	DQ2	66	Yes	No
6	F64	IIIC	DQ2	47	Yes	Yes
7	F69	IIIC	DQ2/2	44	Yes	No
8	F65	IIIA	DQ2	47	Yes	No
9	M46	UJ	Non DQ2/8	33	Yes	No
10	M66	IIIA	DQ2	30	Yes	No
11	M51	IIIA+UJ	DQ2/2	27	Yes	No
12	M62	IIIB	DQ2/2	25	Yes	No
13	F57	IIIB	DQ2	92	Yes	No
14	M65	IIIA	DQ2/2	67	Yes	No
15	F56	IIIB	DQ2	86	No	No
16	M69	IIIB	DQ2	43	Yes	No

Table 1. Patient characteristics of all RCD II patients included in the study

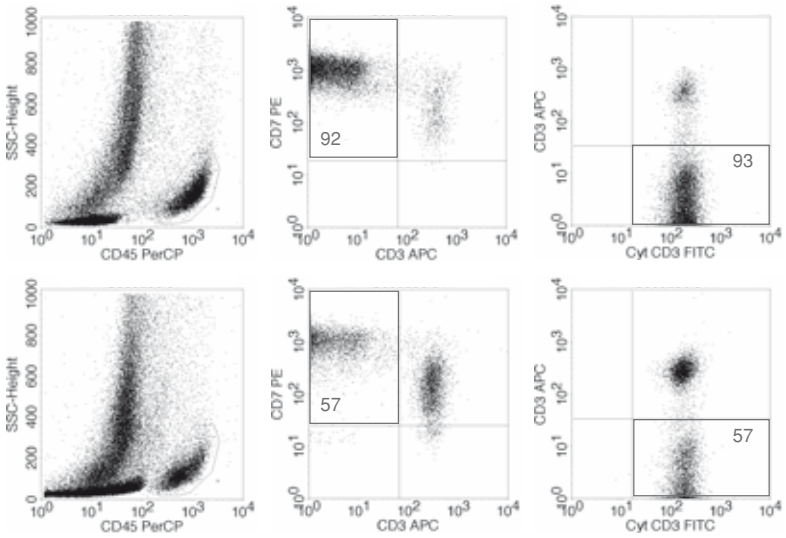

Figure 1. Example of flow cytometric analyses of intestinal lymphocytes isolated from the epithelial as well as the lamina propria layer (patient 1). Upper half: The aberrant IEL (92-93%). Bottom half: The aberrant LPL (57%). Left: Lymphocyte selection gate based on CD45 positivity and low sideward scatter. Middle: The aberrant T-cell population by double staining of surface CD3- CD7+ within the CD45+ cells; Right: surface CD3-, cytoplasmic CD3+ cells, double staining shown within the CD7+/CD45+cells.

Lympho subset (%) Patient	CD3+ T-cells	CD8+ T-cells	CD4+ T-cells	CD7+ Lympho	TCRγδ+ T-cells	CD16/56+ NK-cells	CD19+ B-cells	CD7+ CD3- cytCD3+ aberrant T-cells
1. IEL	8	4	2	99	0.6	1	0.02	93
LPL	46	21	22	96	2	4	0.6	57
2. IEL	26	17	9	99	2	5	0.1	69
LPL	55	20	35	96	1	2	0.6	38
3. IEL	32	11	22	97	2	1	0.3	69
LPL	79	31	50	79	3	10	4	21
4. IEL	46	29	12	99	7	1.3	0.1	60
LPL	84	41	45	90	5	1.8	3	11
5. IEL	35	24	10	99	3	3	1	66
LPL	62	21	46	79	3	4	18	22
6. IEL	7	2	3	30	2	1	0.05	47
LPL	51	10	32	89	5	14	5	20
7. IEL	44	26	18	98	6	25	0.1	44
LPL	75	37	44	87	5	12	3	17
8. IEL	46	36	5	99	12	3	0.06	47
LPL	84	44	39	92	7	3	1	8
9. IEL	37	21	20	98	2	29	0.2	33
LPL	88	71	22	91	5	6	0.3	8
10. IEL	54	42	8	99	6	14	0.06	30
LPL	86	53	32	94	2	8	1	5
11. IEL	70	52	16	96	7	9	0.03	27
LPL	79	39	41	84	9	11	1	12
12. IEL	61	52	14	93	5	1	4	25
LPL	71	31	44	72	2	9	7	14
13. IEL	15	10	4	99	3	7	0	92
LPL	40	9	34	75	2	6	15	36
14. IEL	35	18	12	98	14	3	0.6	67
LPL	75	20	55	92	9	5	2	17
15. IEL	10	4	3	99	3	1	0.3	86
LPL	24	10	15	98	3	1	2	75
16. IEL	54	47	6	100	8	4	0.1	43
LPL	67	50	30	98	6	5	2	31

Table 2. Lymphocyte subsets in duodenal biopsy specimens of RCD II patients as % of intestinal lymphocytes, determined by Flow cytometry. In all 16 patients the intraepithelial lymphocytes (IEL) and the lamina propria lymphocytes (LPL) were analyzed separately. Lympho= lymphocytes

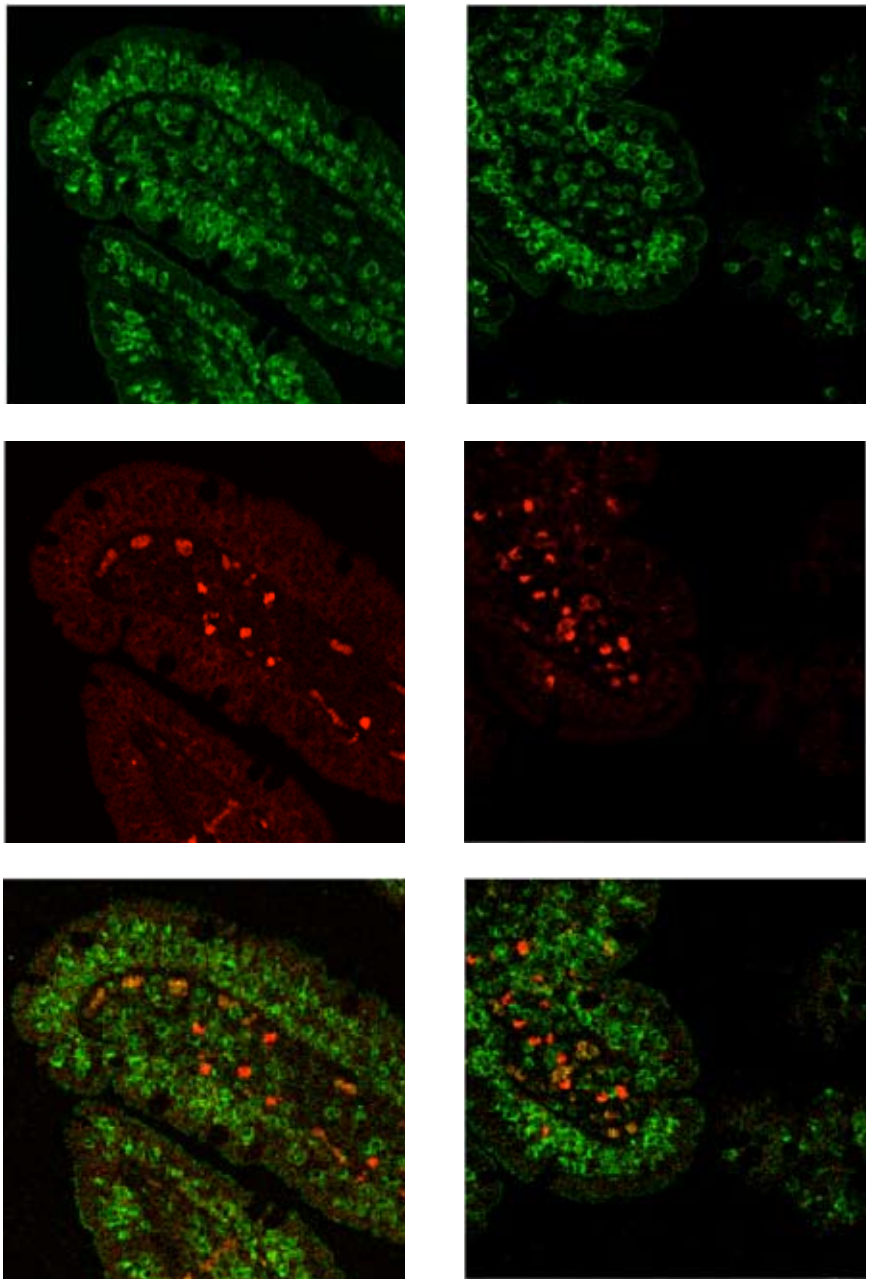

Figure 2. In situ localization of aberrant IEL and LPL (patient 1).
Immunofluorescence analysis of: Upper: Mucosal CD3 positive T-cells visualized by green (FITC) fluorescence Middle: Mucosal CD4 or CD8 positive T-cells visualized by red (TRITC) fluorescence. Bottom: The overlay of staining A and B, showing the presence of both aberrant (green) and normal (orange) intestinal T-cells.(200x)

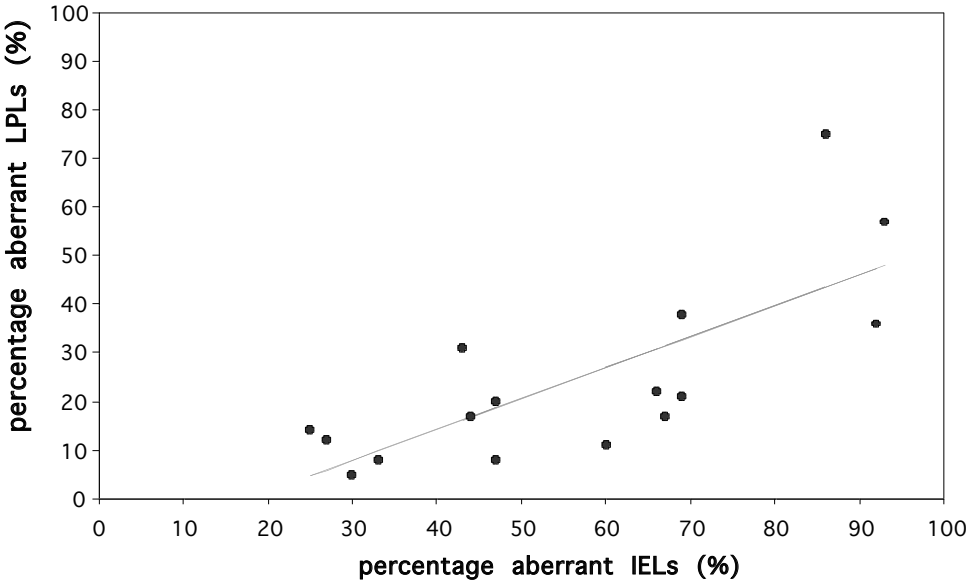

Figure 3. The Percentage aberrant T-cells (CD7+ surface CD3- Cytoplasmic CD3+) in the Epithelial layer (IEL) versus the Lamina propria layer (LPL). Liniar regerssion analysis revealed that a rise in aberrant IELs of 10% results in an increase in aberrant LPL of 6.4% (p=0.001).

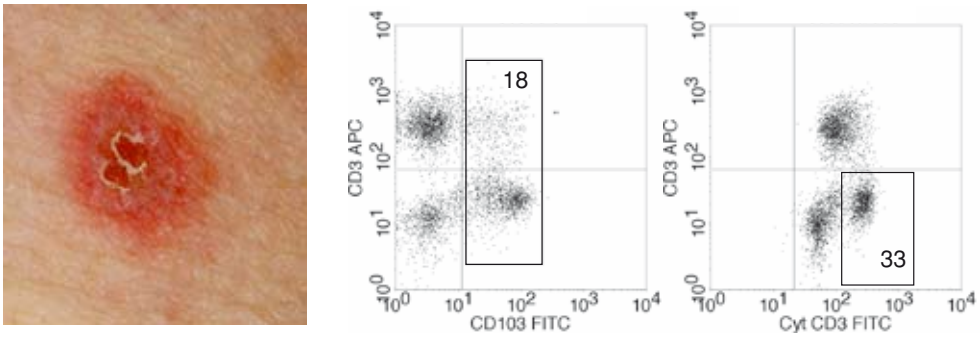

Figure 4. Flow cytometric analyses of lymphocytes isolated from atypical lesions in a RCD II patient (patient 1). Left: a representative skin lesion in RCD II patient no. 1 (approximately 1 cm in diameter). Middle and right: CD3 negative lymphocytes that are CD103 positive, within a lymphocyte selection gate based on CD45 positivity and low sideward scatter. The aberrant surface CD3-, cytoplasmic CD3++ T-cell population in this patient is 33%.

CD3+ CD4/8- lymphocytes, in agreement with flow cytometry. The intraepithelial compartment did not show normal T-cells in this patient, reflecting the 93% aberrant IEL found by flow cytometry.
In **table 2** the percentage lymphocyte subsets in both IEL and LPL fractions in all RCD II patients are shown. Our results clearly show that aberrant T-lymphocytes may be present in both the IEL and the LPL compartments of RCD II derived small intestinal biopsies. Although the highest percentages are always present in the IEL compartment, aberrant LPL can exceed 20% of total LPL in half the RCD II patients.
In order to investigate the relation between aberrant IELs and LPLs, their levels were directly compared within the group of 16 RCD II patients. Linear regression analysis revealed that a rise in aberrant IELs of 10% results in an increase in aberrant LPL of 6.4% ($p=0.001$). **Figure 3** depicts the relationship between the percentages aberrant IELs and LPLs. Since the normal CD8 expression is lost upon progression to an aberrant phenotype there was a negative correlation between the percentage CD8+ IELs and aberrant IELs ($p<0.0005$) as well as the percentage CD8+ LPLs and aberrant LPLs ($p=0.013$).

The distribution of other lymphocyte subsets in the epithelium and lamina propria
As can be seen in **table 2** the percentages TCRγδ T-cells are low in both the intraepithelial layer and the lamina propria. There were no significant differences between the two compartments. As expected, the proportion of CD4+ T-cells was significantly lower in the intraepithelial fraction as compared to the lamina propria fraction($p=0.048$). Although there was no significant difference in percentage of CD19+ B-cells, a clear trend towards a higher percentage within LPL fraction was oberved.

Analysis of aberrant T-cells in RCD II patients with skin lesions
Histopathological evaluation of atypical skin lesions that developed within two RCD II patients indicated a lymphocytic infiltrate, sharing morphologcal characteristics with a cutaneous T-cell lymhoma. Interestingly, cutaneous lymphocytes isolated from these skin lesions and subsequently analyzed by flow cytometry showed a similar aberrant immunophenotype as was found in the intestinal mucosa. The two patients that developed such skin lesions (patient 1 and 6) are shown in **table 1** and a representative example of one of the lesions itself in **figure 4** (left panel). Interestingly, in one patient (patient 6) the skin lesions eventually disappeared spontaneously, whereas patient 1 developed overt cutaneous T-cell lymphomas as well as intestinal EATL. **figure 4** depicts the flow cytometric analysis of lymphocytes isolated from a skin biopsy specimen from patient 1. The presence of CD103 on these cutaneous lymphocytes suggests their derivation from the gut epithelium (18%, middle panel of **figure 4**). Furthermore, a substantial number of these CD103+ lymphocytes were CD3 negative, and lymphocytes with an aberrant (CD3- CD7+ cytCD3++) phenotype could be clearly identified (33%, right panel **figure 4**). Clonality analysis performed on the skin biopsy specimens as well as the duodenal biopsy specimens within the same patient revealed similar monoclonal TCR-γ rearrangement patterns, confrming their shared clonall origin from the gut epithelium.

Discussion

In this study we set out to investigate whether aberrant T-lymphocytes in RCD II can be detected in other parts of the small intestinal mucosa besides the epithelial compartment. Additionally, the presence of aberrant T-lymphocytes was analyzed in two RCD II patients that developed multiple atypical skin lesions.

Previously we have shown that quantification of aberrant T-cells by flow cytometry is preferable to T-cell clonality analysis for identification of RCD patients at risk for EATL development. A cut-off value of 20% aberant T-cells in the intraepithelial compartment is of use in risk stratification of RCD patients. Aberrant IELs were found in RCD II patients with a median of 52% (range 27-94%) and medians of 1-8% in CD patient and control groups11 Little is known about the distribution of these aberrant T-cells thoughout the different compartments of the small intestine. Verkarre et al.13 and Cellier et al 4;8 showed by means of immunohistochemistry that aberrant IELs can be found diffusely spread at different longitudinal levels throughout the intestinal tract. Furthermore, Cellier et al showed their presence in blood by flow cytometry.4;8

The present study clearly shows that aberrant T-lymphocytes can be present in both the IEL and the LPL compartments of RCD II patients. Although the highest percentages are always present in the IEL compartment, aberrant LPL can exceed 20% of total LPL in half the RCD II patients. Aberrant IELs and LPLs showed a significant linear relation: a rise in aberrant IELs of 10% resulted in an increase in aberrant LPL of 6.4% ($p=0.001$). The presence of aberrant LPL was confirmed by immunohistochemical staining, excluding contamination of LPL by IEL during isolation.

The percentages TCRγδ+ T-cells in the present study were low in the epithelial layer as well as in the lamina propria. In a previous study we have also found a significantly lower proportion of TCRγδ+ IELs in RCD II as compared to all other CD groups.30 Regarding the proportion of CD4+ T-cells and CD19+ B-cells, one would expect higher proportions in the lamina propria layer than intraepithelially, as these cells are known to reside in this compartment. As compared to the intraepithelial layer, this was indeed the case for CD4+ T-cells ($p=0.048$). However, for CD19+ B-cells the relation was not significant, possibly due to large variability in proportions in combination with the relatively small sample size.

No previous studies on the cross-sectional small intestinal dissemination of aberrant T-cells in RCD II have been performed sofar. Our results indicate that aberrant LPLs can indeed be detected, indicating cross-sectional dissemination. The close contact of the lamina propria to the blood stream may thus faciliate the ability of aberrant T-lymphocytes to spread to an extraintestinal level. This is an interesting finding as EATLs are known to have the ability to spread extraintestinally.14 Indeed, cutaneous lymphocytes isolated from patients developing atypical skin lesions showed a similar aberrant immunophenotype as was found in the intestinal mucosa. An explanation for the actual presence of aberrant T-cells in the skin is not currently available since we did not analyze the presence of skin homing receptors yet. However, their expression of CD103, in addition to a similar monoclonal TCR rearrangement, at least indicates their origin from gut-derived clonal aberrant T-cells.

Apparently, in RCD II the aberrant T-lymphocytes may also reside in the extraepithelial layer of the small intestinal mucosa, and even in an extraintestinal localization including the skin. Whether this phenomenon represents a passive overflow from the intestinal epithelium or active trafficking towards an other anatomical localization remains to be elucidated. In conclusion, our results show that RCD II indeed is a disseminated disease, which clearly imposes a risk of EATL development outside the intestine. To what extent the dissemination of aberrant T-cells in RCD II contributes to the spreading of EATL has yet to be elucidated. An interesting scope for future investigations would be to investigate whether a high percentage aberrant LPLs is an independent prognostic parameter for the development extraintestinal EATL.

Acknowledgments

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6.
Defective expression of T-cell receptor chains underlies loss of surface T-cell receptor-CD3 expression in Refractory Coeliac Disease type II.

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Abstract

Enteropathy associated T cell lymphoma, an often fatal complication of celiac disease, can result from expansion of aberrant intraepithelial lymphocytes (IELs) in refractory celiac disease type II (RCD II). Aberrant IELs and lymphoma cells are intracellularly CD3ε$^+$, but lack expression of the T cell receptor (TCR)-CD3 complex on the cell surface. It is unknown what causes the loss of TCR-CD3 expression. We report the isolation of a cell line from a RCD II patient with the characteristic phenotype of enteropathy associated T cell lymphoma. We demonstrate that in this cell line the TCRα- and β-chains as well as the CD3γ-, CD3δ-, CD3ε- and ζ-chains are present intracellularly and that assembly of the CD3γε-, CD3δε- and ζζ-dimers is normal. However, dimerization of the TCR-chains and proper assembly of the TCR-CD3 complex is defective. Upon introduction of exogenous TCRβ-chains, but not of TCRα-chains, assembly and functional cell surface expression of the TCR-CD3 complex was restored. Defective synthesis of both TCR-chains was found to underlie loss of TCR expression in similar cell lines isolated from two additional patients. (Pre)malignant transformation in RCD II thus correlates with defective synthesis or defective association of the TCR-chains, resulting in loss of surface TCR-CD3 expression.

Introduction

Coeliac disease (CD) is an inflammatory disorder of the small intestine caused by a dysregulated immune response to ingested wheat gluten, which typically leads to villous atrophy and increased numbers of intraepithelial lymphocytes (IELs) in the intestinal mucosa. While most patients recover on a gluten-free diet, a small proportion of patients fails to improve and develops a condition called refractory celiac disease (RCD). RCD is characterized by persisting or recurring villous atrophy with crypt hyperplasia and an increase of IELs despite a gluten-free diet. Two types of RCD are currently recognized: RCD I, without aberrant IELs and RCD II, with aberrant IELs[1;2;3]. The aberrant IELs in RCD II lack CD3, CD4, CD8 and the T cell receptor (TCR) on the surface, but express CD3 intracellularly and display monoclonal TCR-γ gene rearrangement[2;4;5]. Furthermore, it has been shown that IL15 is upregulated in the lamina propria and epithelial cells of RCD patients which induces growth and activation of these clonal IELs[6;7]. Since an expansion of IELs under the influence of IL15 may eventually give rise to overt enteropathy associated T cell lymphoma (EATL), the presence of such a clonal IEL population is thought to be a premalignant condition [1;2;6;8]. It is not known what drives lymphoma development and the associated loss of TCR expression in RCD II. In the present study we report the isolation of a cell line from a duodenal biopsy of a patient with RCD II. This cell line has the characteristic CD4$^-$, CD8$^-$, intracellular CD3ε$^+$, surface TCR-CD3$^-$ phenotype of RCD II associated IELs and proliferates in the presence of IL15. In addition, these IELs express CD30 on the cell surface, as is typically seen in EATL[9]. We have used this cell line and subunit-specific antibodies to analyze the expression and assembly of the TCR and CD3 subunits. The results indicate that while all TCR-CD3

subunits were present intracellularly, proper assembly of the TCRαβ-dimer was defective. Functional cell surface expression of the complex could be restored by the introduction of an exogenous TCRβ-chain. A similar analysis of cell lines isolated from two additional RCD II patients indicated defects in the synthesis of the TCR-chains. Defective synthesis or defective association of T cell receptor chains thus causes loss of functional surface TCR-CD3 expression on IELs in RCD II, a process which is likely important in escape from immune regulation and progression into enteropathy associated T cell lymphoma.

Materials and methods

Patient histories

Patient 1 (P1) was typed as HLA-A1/A2, -B8, -Cw7/Cw12, DR3/7, DQ2 and developed celiac disease at the age of 51 years. At age 67, refractory celiac disease type II with aberrant IELs was diagnosed. Until the present study no EATL has developed[10]. Patient 2 (P2) was typed as HLA-A3/32, -B8, -Cw7, DR3, DQ2 and Patient 3 (P3) as HLA-A1, -B8, -Cw7, DR3, DQ2. Both patient 2 and 3 were RCD II patients with aberrant IELs and without EATL.

Cell lines

As controls the following cell lines were used: T cell clone N10[11], a $CD4^+$ gliadin specific T cell clone isolated from a duodenal biopsy from a celiac disease patient and Jurkat clones deficient for either TCRα ($\alpha^{-/-}$) or TCRβ ($\beta^{-/-}$) expression[12]

Small intestinal biopsy specimens

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe®) were taken from the second part of the duodenum[13]. Biopsy specimens were taken for direct flow cytometric analysis, TCR gene rearrangement assessment and T cell culture. All biopsy specimens were obtained after given informed consent in accordance with the local ethical guidelines.

Isolation of intestinal lymphocytes and flow cytometry

Intraepithelial lymphocytes were isolated from duodenal biopsies as described by Madrigal et al [14] with minor modifications. Briefly, biopsies were vigorously shaken at 37°C for 60 min in phosphate buffered saline (PBS) supplemented with 1mM DTT (Fluka BioChemika, Buchs, Switzerland) and 1mM EDTA (Merck, Darmstadt, Germany). The released IELs were washed twice with PBS supplemented with 0,1% BSA (Roche Diagnostics, Mannheim, Germany) and subsequently stained for 30 minutes on ice, with fluorochrome-labeled monoclonal antibodies (MoAbs) directed against CD3, CD4, CD8, CD7, CD45, γδTCR (all from BD Biosciences, San Jose, California) as previously described[15]. Cytoplasmic staining of CD3 was performed after cell permeabilization (Cytofix/CytoPerm Plus™ kit by BD Biosciences). Flow cytometric acquisition was performed using Cellquest software on a Fluorescence Activated Cell Sorter (FACSCalibur, BD Biosciences). The data were analyzed using Cellquest software (BD

Biosciences). All analyses were performed on lymphocytes, based on CD45 ^{bright} staining and low sideward scatter.

Assessment of T-cell clonality

T cell receptor-gamma (TCR-γ) gene rearrangements were analyzed on two cryopreserved biopsy specimens and on cell line P1 (see below). DNA was extracted from cryosections using proteinase-K digestion and ethanol precipitation of the genomic DNA. TCR-γ gene rearrangements were subsequently analyzed by multiplex polymerase chain reaction (PCR) amplification, using the primers and probes provided by the BIOMED-2 consortium according to their guidelines[16].

T cell culture

Intraepithelial and lamina propria T-lymphocytes were isolated from a duodenal biopsy from a RCD II patient. After treatment with 1mM DTT (2 times for 10 min at room temperature) and 0.75 mM EDTA (60 min at 37°C) the biopsy was cultured in IMDM (Lonza, Verviers, Belgium) supplemented with 10% NHS, 10 ng/ml recombinant IL15 (R&D systems, UK), gliadin and gliadin treated with tissue transglutaminase. From day five, cells were further expanded in IMDM with 10% NHS containing 10 ng/ml IL15. Staining of *cells* from T cell culture was performed with fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, TCRαβ, TCRγδ, CD103, integrin β7, CD30 (all from BD Biosciences), NKG2D (R&D) and KIR2DL2/KIR2DL3/KIR2DS2 (MoAb GL183, Beckman Coulter). The predominant cell population consisting of TCRαβ$^-$, CD3$^-$, CD4$^-$, CD8$^-$, CD30$^+$ cells was purified by FACS and cultured in IMDM with 10% NHS containing 10 ng/ml IL15. Cells were restimulated approximately every 6-7 weeks with 1µg/ml PHA, 10ng/ml IL15 and 1x10^6/ml irradiated allogenous PBMC as feeder cells. Stability of the aberrant IEL phenotype of the P1 line was checked with flowcytometry at least once between every two restimulations.

Proliferation Assay

Cells from RCD cell line P1 were rested by culturing them in the absence of IL-15 for four days. Cells (10.000 cells per well) were subsequently cultured in triplicate in 96-well plates in the presence or absence of IL15 and/or IL2 for two to five days at 37°C, after which 0.5 µCi of ^{3}H-thymidine was added to every well. After overnight incubation at 37°C, cells were harvested (Tomtec harvester, Hamden, Connecticut) and ^{3}H-thymidine incorporation was determined.

Antisera and antibodies

The antisera against TCRα-chain, TCRβ-chain, CD3γ-chain, CD3δ-chain and CD3ε-chain were rabbit anti-peptide antisera. As described previously[17;18], the antiserum against the TCRα-chain was raised against a sequence in the extracellular constant region, while antisera against TCRβ-chain, CD3γ-chain, CD3δ-chain and CD3ε-chain were raised against peptides corresponding to the carboxy termini of these chains. The anti-ζ-chain monoclonal antibody was obtained from BD pharmingen. The anti-CD3

antibody OKT3 used in the stimulation assay was obtained from Orthobiotech (Bridgewater, New Jersey).

^{35}S Metabolic Labeling and Cell Surface Iodination

Metabolic labeling and cell surface iodination were performed as previously described [19]. For ^{35}S metabolic labeling, $10x10^6$ cells were washed thrice in PBS and resuspended in 5 ml methionine- and cysteine-free RPMI (Sigma-Aldrich, Zwijndrecht, The Netherlands) containing 0.5% FCS and 10 ng/ml recombinant human IL15. 1 mCi of ^{35}S-methionine/cysteine (NEN) was added and cells were incubated overnight at 37°C. After incubation cells were washed in PBS and lysed overnight at 4°C in 1-2 ml of lysisbuffer containing either 0.5% NP40 (Pierce, Rockford, Illinois) or 1% digitonin (Sigma-Aldrich). For iodination approximately $6x10^6$ cells were washed in PBS and resuspended in 30µl lactoperoxidase solution (2mg/ml, Sigma-Aldrich). 1 mCi Na^{125}I (NEN) was added to the cells followed by the addition of 10µl 0.05% H_2O_2/PBS solution. During frequent mixing 0.05% H_2O_2/PBS solution was added after 5 min (15 µl) and after 15 min (20 µl). After 30 min, free iodine was removed by washing with PBS. Cells were lysed overnight at 4°C in 250-500 µl lysisbuffer containing 1% digitonin.

Immunoprecipitation and SDS-PAGE Analysis

^{125}I or ^{35}S lysates were centrifuged at maximum speed for 20 min in an eppendorf centrifuge at 4°C. Lysates were precleared twice, first with 100 µl protein A sepharose beads and 50 µl normal rabbit serum and second with 100 µl protein A sepharose beads only, both under rotation for 1 hour at room temperature. After removal of the beads, 5 µl antiserum (TCRα-, TCRβ-, CD3γ-, CD3δ-, CD3ε-antisera, anti-ζ-antibody or normal rabbit serum) was added to 100 µl precleared lysate and rotated for 1 hour at room temperature. Antigen-antibody complexes were isolated with 12.5 µl protein A sepharose beads during 1 hour rotation at room temperature. Beads were washed four times in lysisbuffer and analyzed under either reducing or nonreducing conditions on a one-dimensional 13.5% SDS-PAGE gel. After drying of the gels, autoradiography was performed at -80°C using Fuji scientific imaging films (Fuji, Düsseldorf, Germany).

Retroviral Transduction

The TCRα- and TCRβ-chains isolated from T cell clone N10 (TCRAV14, TCRBV4) were cloned into a bicistronic vector as described before[20]. The vector containing the TCRα-chain was combined with the marker green fluorescent protein (GFP), the vector containing the TCRβ-chain was combined with truncated nerve growth factor receptor (tNGFR). Both α- and β-chain constructs were transfected into Phoenix packaging cells[21]. Retroviral supernatant was produced and used to transduce cells from RCD cell lines P1, P2 and P3 with either TCRα, TCRβ or both. For transduction, non tissue culture treated 24 well plates (Falcon, BD Biosciences) were treated 2 hours with 25 µg/ml Retronectin (Takara, Otsu, Japan) and blocked 30 min with 2% HSA. After 30 min

incubation with the cells, retroviral supernatant was added and cells were placed overnight at 37°C. Presence of the TCRα-chain (GFP) and TCRβ-chain (tNGFR) and cell surface expression of the TCR was analyzed with flow cytometry. TCRαβ$^+$, NGFR$^+$ cells were purified by FACS.

T cell stimulation with anti-CD3

96 well non tissue culture treated plates (Falcon, BD Biosciences) were coated overnight in triplicate with varying concentrations of anti-CD3 (OKT3). After coating, plates were blocked with 10% FCS/PBS and washed three times with PBS. Per well 15.000 nontransduced P1-cells, P1-cells transduced with the TCRβ-chain from T cell clone N10 or cells from T cell clone N10 were added. Plates were incubated at 37°C for 2 days, after which 0.5 μCi of ^{3}H-thymidine was added. After overnight incubation at 37°C, cells were harvested and ^{3}H-thymidine incorporation was determined.

Results

The majority of IELs in the small intestine of RCD II patient P1 are aberrant and monoclonal

RCD II is associated with aberrant IELs lacking CD3, CD4, CD8 and the TCR on the cell surface, but expressing CD3 intracellular. To gain insight in the phenotype of the IELs in the small intestine of RCD II patient P1, FACS analysis was performed on IELs directly isolated from a freshly taken duodenal biopsy of patient P1. Analysis of the CD45^{bright} IEL population showed that the majority (71-76%) were, CD3$^-$, CD4$^-$, CD8$^-$, CD7$^+$ but cytoplasmic CD3$^+$, and may thus be defined as aberrant (**Figure 1A**). Furthermore, PCR analysis demonstrated the presence of monoclonal TCR-γ gene rearrangement in two cryopreserved biopsy specimens of patient P1 (data not shown).

RCD cell line P1: a model for aberrant IEL in RCD II and EATL

Culture of a duodenal biopsy from RCD II patient P1 with IL15 resulted in outgrowth of a cell line in which the predominant population, similar to the freshly isolated IELs, was found to be CD3$^-$, TCRαβ$^-$, CD4$^-$, CD8$^-$ and cytoplasmic CD3$^+$ (**Figure 1B**). In addition, these cells were CD30$^+$, which is a characteristic feature of EATL[9] (**Figure 1B**). Furthermore, the cells displayed the same monoclonal TCR-γ gene rearrangement as the two cryopreserved biopsy specimens of patient P1 (data not shown). The predominant population, hereafter called RCD cell line P1, was purified by FACS and subsequently used as a model for aberrant IELs in RCD II and EATL. As IL15 is upregulated in the lamina propria and epithelial cells of RCD patients and induces growth and activation of clonal IEL[6;22], the effect of IL15 on the proliferation of RCD cell line P1 was assessed. **Figure 2A** shows dose-dependent proliferation of RCD cell line P1. Proliferation in response to high doses of IL2 was much lower than proliferation in response to IL15 (**Figure 2B**). Furthermore, combining IL15 and IL2 had no additional effect on proliferation compared to IL15 alone (**Figure 2B**). The specific response of RCD cell line P1 to IL15 further supported the notion that this cell line can serve as a model for aberrant IELs in RCD II and EATL.

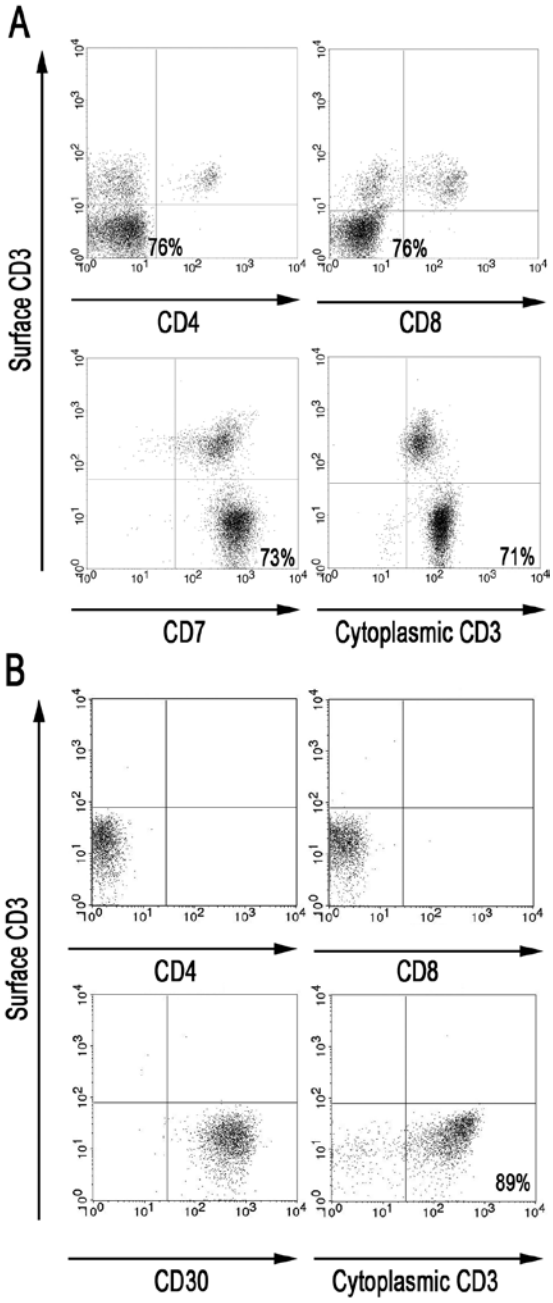


Figure 1. The majority of IELs in the small intestine of RCD II patient P1 are aberrant. (A) FACS analysis of IELs, directly isolated from duodenal biopsies from patient P1. (B) FACS analysis of RCD cell line P1, cultured from duodenal biopsies from patient P1. Analyses were performed on CD45 ^{bright} cells within a live lymphocyte gate.

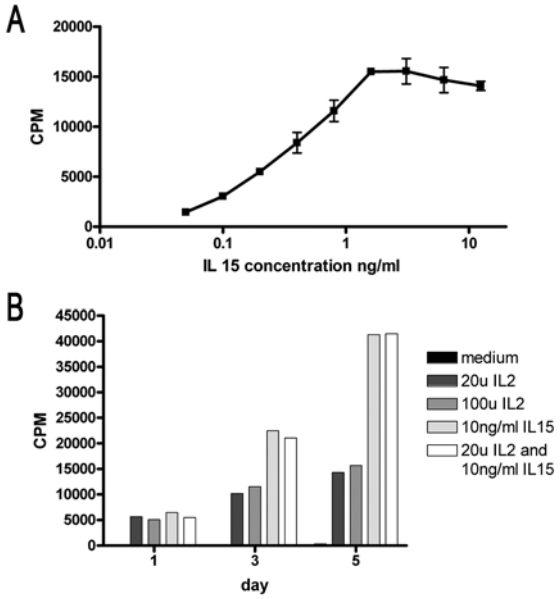


Figure 2. IELs from RCD cell line P1 proliferate in response to IL15 in a dose-dependant manner. (A) Proliferation in response to varying doses of IL15. (B) Five day follow up of proliferation in response to IL2, IL15 and a combination of both. CPM indicates 3H-thymidine incorporation.

Defective association of the TCR-chains underlies the loss of surface TCR-CD3 expression in RCD cell line P1

As a first step to understand why TCR-CD3 surface expression is lost on IELs in RCD II, we investigated the presence or absence of the specific TCR-CD3 chains in RCD cell line P1. For this purpose, cells were labeled with ^{35}S methionine/cysteine and lysed, after which immunoprecipitations with antibodies specific for TCRα, TCRβ, CD3γ, CD3δ, CD3ε and ζ were performed, followed by SDS-PAGE analysis. For comparison, metabolic labeling and immunoprecipitations were performed on cells from unrelated T cell clone N10 which expresses a normal TCR-CD3 complex on the surface. It is known that in digitonin lysis buffer all components of the TCR-CD3 complex remain complexed while in NP40 lysis buffer the TCR-CD3 complex dissociates into the TCRαβ-heterodimer, ζ-homodimer, a CD3γε-heterodimer and a CD3δε-heterodimer[17;19]. RCD cell line P1 was found to express all TCR-CD3 subunits intracellularly (**Figure 3A**). The TCRβ-antibody immunoprecipitated two specific bands that appeared to be glycosylation variants of each other as, in agreement with previous observations[23], upon deglycosylation with N-glycanase, the upper band merged with the lower band (results not shown). Furthermore, the dimerization of ζ appeared normal since under non-reducing conditions ζζ was seen as a protein band of approximately 30 kD which was reduced to a protein band of approximately 15 kD under reducing conditions (**Figure 3A**). In addition, a number of other subunit interactions were observed as indicated by the presence of CD3ε in the CD3γ and CD3δ specific immunoprecipitates and vice versa. Under

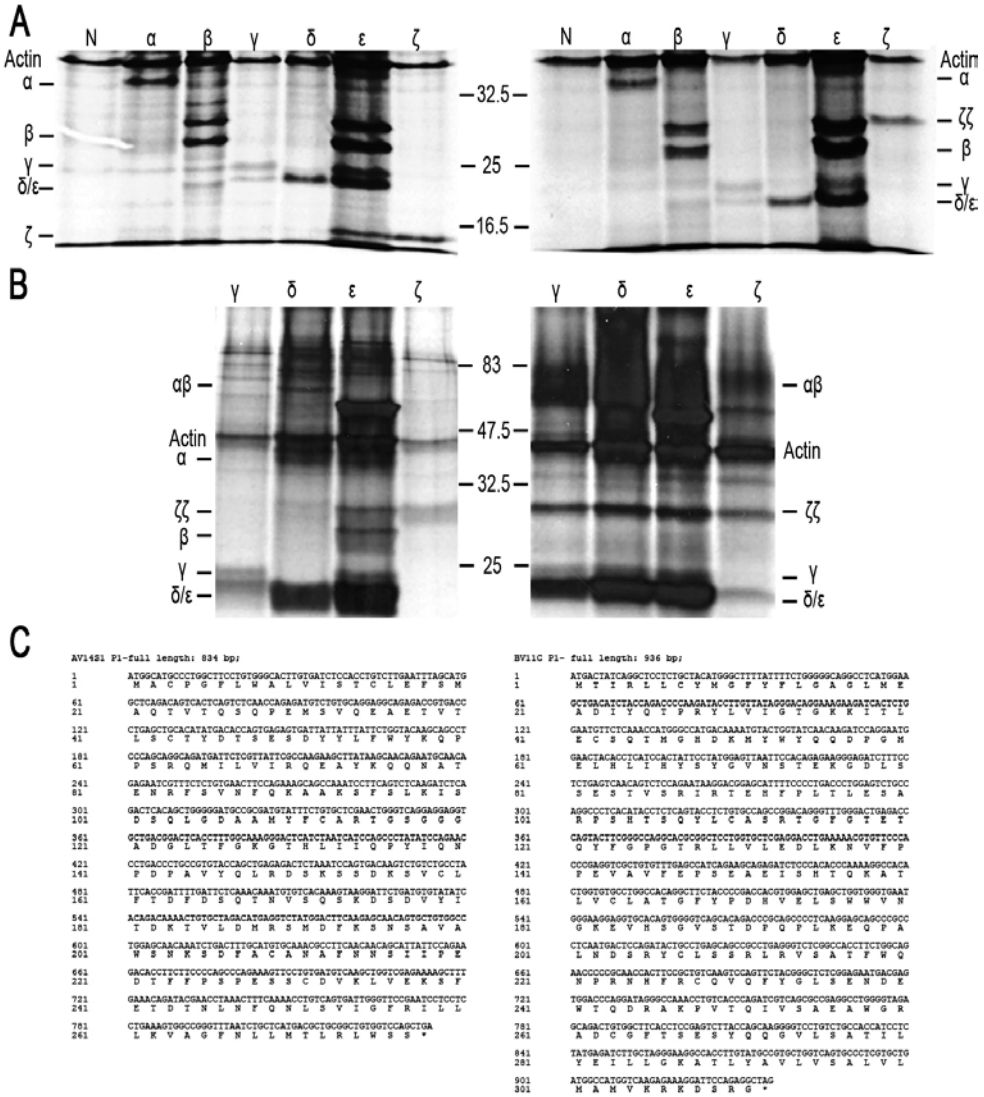


Figure 3. Defective association of the TCR-chains underlies the loss of surface TCR-CD3 expression in RCD cell line P1. SDS-PAGE analysis of immunoprecipitates of RCD cell line P1 and T cell clone N10 after ^{35}S metabolic labeling. Antisera used were normal rabbit serum as negative control (N), anti-TCRα (α), anti-TCRβ (β), anti-CD3γ (γ), anti-CD3δ (δ), anti-CD3ε (ε) and anti-ζ (ζ). (A) immunoprecipitates obtained from NP40 lysates of RCD cell line P1 were analyzed under reducing (left) and non-reducing (right) conditions. (B) immunoprecipitates obtained from digitonin lysates of RCD cell line P1 (left) and T cell clone N10 (right) were analyzed under non-reducing conditions. Positions of individual chains and molecular mass markers (kD) are indicated. (C) Sequences of the TCRα- and TCRβ-chain of cell line P1 (TCRAV14S1, TCRBV11c)

nonreducing conditions, however, there was no evidence for proper formation of a TCRαβ-dimer since only separate TCRα- and β-chains were observed in the TCRα- and TCRβ-specific immunoprecipitates. This suggests the lack of formation of a disulfide bridge between the TCR-chains (**Figure 3A**). The lack of proper formation of a TCRαβ-dimer was further substantiated by analysis of CD3- and ζ-specific immunoprecipitations performed on digitonin lysates of RCD cell line P1 and, as a control, surface TCR^+ T cell clone N10. In RCD cell line P1 only separate TCRα- and TCRβ-chains were observed in association with CD3ε while in T cell clone N10 a TCRαβ-dimer was observed while separate TCRα- and TCRβ-chains were absent (**Figure 3B**). Furthermore, while in all three CD3 subunit-specific immunoprecipitates of T cell clone N10 the ζζ-dimer was present, no evidence for incorporation of the ζζ-dimer into the TCR-CD3 complex was obtained for RCD cell line P1 (**Figure 3B**). Sequencing of the TCRα- and TCRβ-transcripts of RCD cell line P1 revealed that both chains were in frame and encoded full-length TCR-chains (**Figure 3C**). In conclusion, all TCR-CD3 subunits are present in RCD cell line P1 but proper assembly of the complex is disturbed.

Retroviral introduction of exogenous TCRβ-chains in RCD cell line P1 restores TCRαβ-dimer formation and cell surface expression

As the formation of TCRαβ-dimers was found to be disturbed in RCD cell line P1, we next investigated whether the introduction of an exogenous TCRα- and/or TCRβ-chain could restore cell surface expression of the TCR-CD3 complex. To this end, a TCRα- and/or

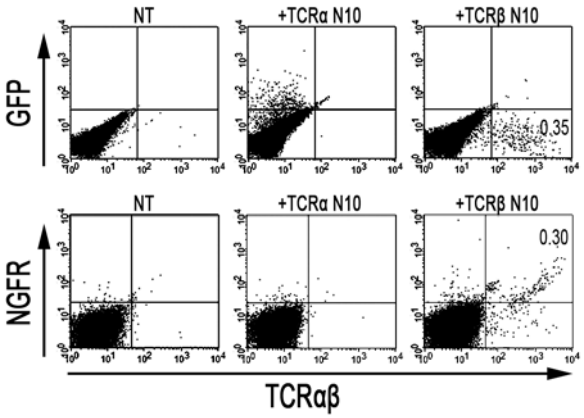
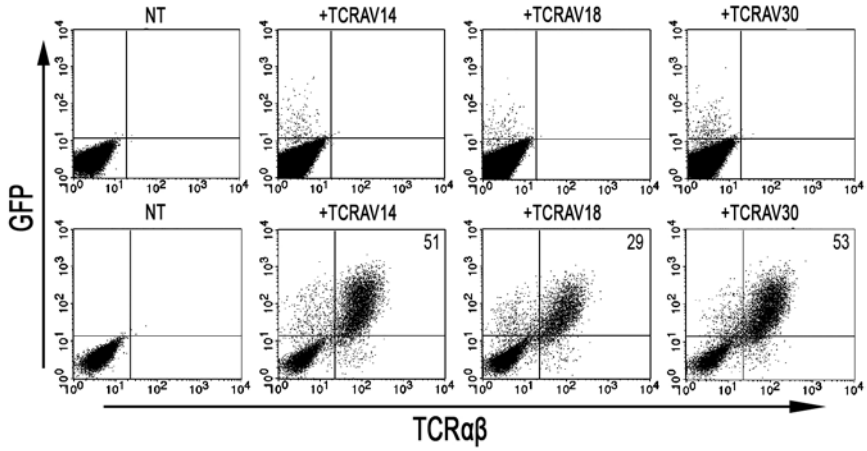


Figure 4. Retroviral introduction of exogenous TCRβ-chains in RCD cell line P1 restores surface TCRαβ expression. FACS analysis after retroviral transduction of cells from RCD cell line P1 with the TCRα- or TCRβ-chain from T cell clone N10. The upper panel shows GFP- (TCRα from N10) and TCRαβ-expression on nontransduced P1-cells (NT), P1-cells transduced with the TCRα-chain from T cell clone N10 (+ TCRα N10) and on P1-cells transduced with the TCRβ-chain from T cell clone N10 (+ TCRβ N10). The lower panel shows NGFR- (TCRβ from N10) and TCRαβ-expression, also on nontransduced P1-cells (NT), P1-cells transduced with the TCRα-chain from T cell clone N10 (+ TCRα N10) and on P1-cells transduced with the TCRβ-chain from T cell clone N10 (+ TCRβ N10)



Supplementary data

Figure S1. Retroviral introduction of exogenous TCRα-chains does not restore surface TCRαβ expression on cell line P1. FACS analysis after retroviral transduction of cells from RCD cell line P1 and a Jurkat TCRα-negative clone with TCRAV14, TCRAV18 and TCRAV30. GFP expression (successful TCRα introduction) is plotted against surface TCRαβ expression. The upper panel shows nontransduced (NT) P1 cells and P1 cells transduced with TCRAV14, TCRAV18 and TCRAV30. The lower panel shows nontransduced (NT) TCRα-negative Jurkat cells and TCRα-negative Jurkat cells transduced with TCRAV14, TCRAV18 and TCRAV30. Percentages of double positive cells are indicated

TCRβ-chain (TCRAV14, TCRBV4) obtained from T cell clone N10, was introduced into RCD cell line P1 by retroviral transduction. For comparison, the exogenous TCRα- or TCRβ-chains were also retrovirally transduced into Jurkat clones deficient for TCRα ($\alpha^{-/-}$) or TCRβ ($\beta^{-/-}$). Cell surface expression of the TCR after transduction was determined with FACS analysis, where GFP-positivity represented proper transduction of the TCRα-chain and NGFR-positivity proper transduction of the TCRβ-chain (**Figure 4**). TCR-CD3 expression was restored on TCRα-negative Jurkat cells after transduction with the TCRα-chain (supplementary data, **figure S1**). Similarly, TCR-CD3 expression was restored on TCRβ-negative Jurkat cells after transduction with the TCRβ-chain (data not shown), indicating that both constructs are functional. While introduction of the TCRα-chain did not restore TCR-CD3 expression on the cell surface of RCD cell line P1, cells transduced with the TCRβ-chain did express the TCR-CD3 complex on the cell surface (**Figure 4**). Similarly, the introduction of two additional TCRα-chains (supplementary data, **figure S1**) failed to restore TCR-CD3 expression on the P1 cells while the introduction of another TCRβ-chain (data not shown) did restore TCR-CD3 expression. To further substantiate that the introduction of an exogenous TCRβ-chain resulted in proper assembly and cell surface expression of a TCR-CD3 complex, $TCR\alpha\beta^{+}$, $NGFR^{+}$ cells were purified by FACS from P1-cells transduced with the TCRβ from N10 (**Figure 4**). Subsequently, T cell clone N10, RCD cell line P1 and RCD cell line P1 transduced with

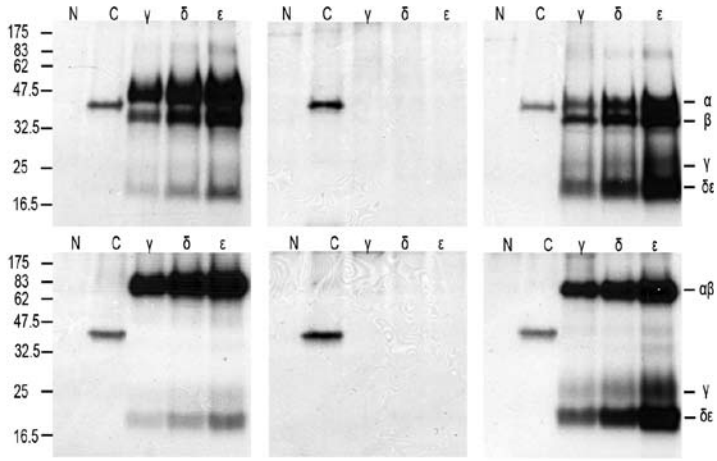


Figure 5. Retroviral introduction of exogenous TCRβ-chains in RCD cell line P1 restores TCRαβ-dimer formation and surface expression. SDS-PAGE analysis of immunoprecipitates obtained from digitonin lysates of T cell clone N10 (left), RCD cell line P1 (middle) and RCD cell line P1 transduced with TCRβ from N10 (right) after cell surface iodination. Antisera used were normal rabbit serum as negative control (N), anti-HLA class I (C), anti-CD3γ (γ), anti-CD3δ (δ) and anti-CD3ε (ε). The upper panel shows reducing conditions, the lower panel nonreducing conditions. Positions of individual chains and molecular mass markers (kD) are indicated.

TCRβ from N10 were either cell surface labeled with ^{125}I, or metabolically labeled with ^{35}S methionine/cysteine. Thereafter, cells were lysed in digitonin buffer to preserve subunit interactions followed by immunoprecipitations and SDS-PAGE analysis. In the metabolically labeled cells the introduction of the TCRβ-chain resulted in the formation of a TCRαβ-dimer and proper assembly of a TCR-CD3 complex, including incorporation of the ζζ-dimer into the complex (data not shown). Similarly, a CD3-associated TCRαβ-dimer was observed after cell surface labeling on T cell clone N10 as well as on RCD cell line P1 transduced with the TCRβ-chain but not on RCD cell line P1 itself (**Figure 5**). Proper assembly was further indicated by the presence of a TCRαβ-dimer in the CD3γ-, CD3δ- and CD3ε-immunoprecipitates and by dissociation of the TCRαβ-dimer into its subunits under reducing conditions (**Figure 5**). Together, these results indicate that impaired dimerization with the endogenous TCRβ-chain results in the loss of a functional TCR-CD3 complex on RCD cell line P1.

Restoration of TCR functionality upon introduction of an exogenous TCRβ-chain in RCD cell line P1

To investigate whether the TCR-CD3 complex expressed on the cell surface after introduction of an exogenous TCRβ-chain was functional, we stimulated T cell clone N10, RCD cell line P1 and the RCD cell line P1 transduced with the TCRβ-chain from N10

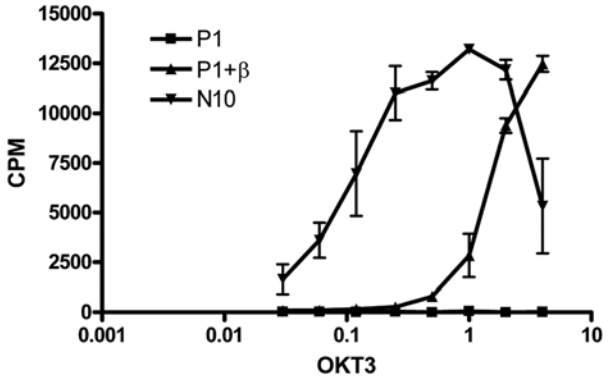


Figure 6. Restoration of TCR functionality upon introduction of an exogenous TCRβ-chain in RCD cell line P1. Cells from T cell clone N10, nontransduced P1-cells and P1-cells transduced with the TCRβ-chain from T cell clone N10 were stimulated with varying amounts of anti-CD3 antibody (OKT3).

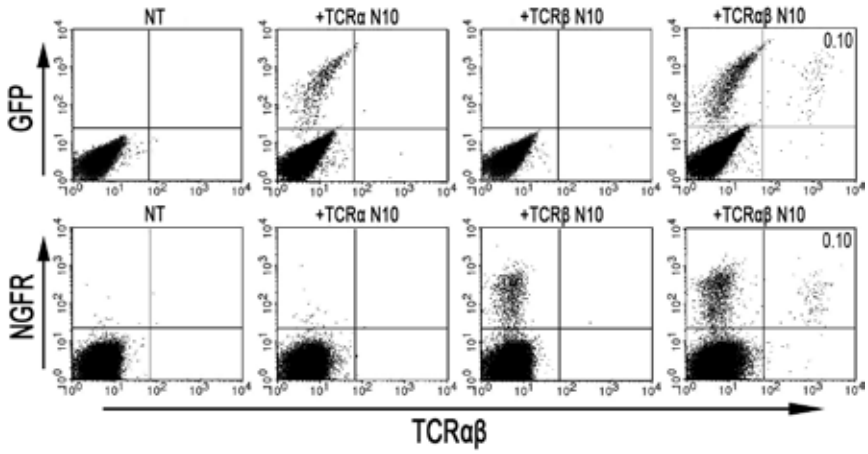


Figure 7. Retroviral introduction of both an exogenous TCRα- and TCRβ-chain in RCD cell lines P2 and P3 restores TCRαβ surface expression. FACS analysis after retroviral transduction of cells from RCD cell line P3 with the TCRα- and/or TCRβ-chain from T cell clone N10. The upper panel shows GFP- (TCRα from N10) and TCRαβ-expression on nontransduced P3-cells (NT), P3-cells transduced with the TCRα-chain from T cell clone N10 (+ TCRα N10), P3-cells transduced with the TCRβ-chain from T cell clone N10 (+ TCRβ N10) and P3-cells with both the TCRα- and TCRβ-chain from T cell clone N10 (+ TCRαβ N10). The lower panel shows NGFR- (TCRβ from N10) and TCRαβ-expression, also on nontransduced P3-cells (NT), P3-cells transduced with the TCRα-chain from T cell clone N10 (+ TCRα N10), P3-cells transduced with the TCRβ-chain from T cell clone N10 (+ TCRβ N10) and P3-cells with both the TCRα- and TCRβ-chain from T cell clone N10 (+ TCRαβ N10). Percentages of double positive cells are indicated. FACS analysis after retroviral transduction of cells from RCD cell line P2 with the TCRα- and/or TCRβ-chain from T cell clone N10 showed similar results.

with a plate bound anti-CD3 antibody (OKT3) and determined the proliferative response. As expected, the nontransduced cells did not respond to anti-CD3 stimulation while the TCRβ-transduced cells proliferated in response to anti-CD3 stimulation, although to a lower extent than control cell line N10 (**Figure 6**). These results show that functional cell surface expression of a TCR-CD3 complex can be restored by introduction of an exogenous TCRβ-chain.

Additional RCD cell lines show impaired TCRα- and TCRβ-dimerization as well

In two additional surface TCR-CD3 negative cell lines isolated from duodenal biopsies from RCD type II patients (P2 and P3), the TCR-CD3 complex was studied. Similar to RCD cell line P1, cells from cell line P2 and P3 proliferated specifically in response to IL15 and ^{35}S metabolic labeling experiments indicated proper assembly of the CD3γε-, CD3δε- and ζζ-dimers while no evidence for the presence of a TCRαβ-dimer was obtained (data not shown). While transduction with either the TCRα- or TCRβ-chain of T cell clone N10 did not restore cell surface expression of the TCR, simultaneous introduction of both TCR-chains did restore TCR surface expression (**Figure 7**), confirming that the CD3 complex assembles properly in these cell lines. In contrast to cell line P1, no transcripts coding for either a TCRα- or a TCRβ-chain could be detected in cell lines P2 and P3 (results not shown). These results indicate that loss of surface TCR-CD3 expression in RCD II cell line P2 and P3 is due to defects in the synthesis of both TCR-chains.

Discussion

Coeliac disease is a common gastrointestinal disorder which afflicts 1 in 200 persons in Europe[24]. 2-5% of celiac disease patients diagnosed as adults, develops a refractory state of celiac disease characterized by persisting villous atrophy and an increase of IELs despite a gluten-free diet [2]. The two types of RCD, RCD I and RCD II, are distinguished by the respective absence or presence of an aberrant IEL population lacking surface TCR-CD3 expression. In all patients with RCD II this abnormal IEL population may be observed, which besides lack of T cell markers such as CD3, CD4, CD8 and TCRαβ is also associated with clonal TCR-γ gene rearrangement[1;2]. Moreover, the aberrant IEL population is not restricted to the small intestine, but may also be observed in gastric and colonic epithelium[1;25]. The same aberrant IELs and clonal TCR-γ gene rearrangements found in patients with RCD II may be subsequently observed in EATL-specimens from these patients, suggesting that RCD II precedes development of EATL [5]. EATL has a very poor 5-year survival rate of 11-20% [2]. Improved understanding of the events leading to RCD II and subsequent EATL development is therefore needed. Until now aberrant IELs have been investigated mainly in situ and this limits the type of experiments that can be performed to investigate molecular events that are linked to malignant transformation[15].

In the present study we report the isolation of three cell lines from small intestinal biopsies of RCD II patients (P1-P3). These cell lines displayed the characteristic intracellular $CD3\varepsilon^+$, surface $CD3^-$, $CD4^-$, $CD8^-$ and $TCR\alpha\beta^-$ phenotype. Moreover, the observed

proliferative response of the cell lines to stimulation with IL15 supports the notion that these cell lines represent a model for aberrant IEL. Strikingly, and in contrast to aberrant IELs in RCD II, the cell lines also expressed CD30 which is typically found on EATL. The latter suggests that while clinically there was no evidence for EATL in these three patients, cells with the characteristic EATL phenotype are already present in the small intestine of these patients and can be propagated in vitro. These cell lines offered the unique opportunity to study the cause for loss of surface expression of the TCR-CD3 complex, an event that is typically associated with (pre)malignant transformation. It is well established that in a functional TCR-CD3 complex the TCRα- and TCRβ-chain are associated with the CD3γ-, CD3δ-, CD3ε-, and ζ-chains, which enable signal transduction[18;26]. Therefore we hypothesized that loss of TCR-CD3 expression might be due to defects in one of these chains resulting in deficient assembly of the complex. We demonstrate that in RCD II cell line P1 the TCRα- and β-chains as well as the CD3γ-, CD3δ-, CD3ε- and ζ-chains were present intracellularly, that the CD3γε-, CD3δε- and ζζ-dimers assembled normally, but that dimerization of the TCRα- and β-chains and incorporation of ζζ in the TCR-CD3 complex was defective. Furthermore, we demonstrate that through the introduction of exogenous TCRβ-chains, but not of TCRα-chains, the assembly and functional cell surface expression of the T cell receptor-CD3 complex could be restored, indicating that the defect lies with the TCRβ chain. Sequencing of the cDNA encoding the endogenous TCRα- and TCRβ-chain from RCD cell line P1 showed both chains to be in frame and according to the consensus sequence (**Figure 3C**), which correlates with the observed presence of a TCRβ-protein in metabolically labeled P1-cells (**Figure 3A**). In contrast, our results indicate that a lack of expression of both TCR-chains underlies the loss of surface TCR-CD3 expression in cell lines P2 and P3. Therefore, our results indicate that the loss of TCR-CD3 expression in patients with RCD can be mediated by several mechanisms. At present it can not be excluded that cell lines P2 and P3, in which both TCR-chains are lost, represent a more advanced stage of (pre)malignant transformation, whereas the absence of association despite the presence of wildtype TCR chains in P1, might represent an earlier phase. This possibility and the exact mechanism underlying defective assembly in RCD cell line P1 will be the subject of future investigations. Preliminary experiments indicate that the half-lives of the TCRα- and TCRβ-chains of cell line P1 are comparable to those in a T cell clone with normal TCR surface expression. Together, our results indicate that (pre)malignant transformation of IEL in RCD II correlates with abnormal expression or association of the TCR-chains, resulting in defective TCR-CD3 surface expression.

As the loss of TCR-CD3 expression is typically observed in RCD II and EATL, one must assume that this is linked to (pre)malignant transformation[5;27]. An important question, therefore, is what drives the downregulation of the TCR-CD3 complex. Downregulation of TCR expression has been linked to extensive stimulation with antigen presented by antigen presenting cells and serves to prevent apoptosis induction[28;29]. Possibly, the aberrant IELs in RCD II express autoreactive TCR or TCR reactive with (peptides from) stress induced ligands. Downregulation of the TCR might then be a way to escape from immune regulatory processes aimed at the elimination of autoreactive cells.

Alternatively, aberrant IELs might arise from gluten specific, HLA-class I restricted $CD8^+$ IELs[30] which escape from immune regulation by downregulation of their gluten-specific TCR. The availability of the IEL cell lines established in the present study now allows an in depth analysis of these possibilities and this will be the topic of future research.
In conclusion, the present study provides the first evidence that loss of TCR-CD3 surface expression on IELs in RCD II is due to defects in the synthesis or assembly of T cell receptor chains providing a first step in understanding the process leading to the development of RCD II and subsequent progression into EATL.

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Authorship

Contribution: J.M.L.T. performed the research, analyzed the data and wrote the paper; W.H.M.V performed experiments and wrote the paper; Y.M.C.K-W, B.H.N, A.R.vd.S, A.T, M.W.J.S and L.H.A.D. performed experiments; C.J.M contributed the duodenal biopsies; M.H.M.H and J.v.B designed the research; F.K designed the research and wrote the paper
Conflict of interest disclosure: The authors declare no competing financial interests
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7.
The presence of intestinal intra-epithelial gamma/delta T-lymphocytes is inversely correlated with lymphoma development in Refractory Coeliac Disease.

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Abstract

Background: In Refractory Coeliac Disease (RCD) type II, a phenotypically aberrant (CD7+CD3-CD4/8-cytoplasmicCD3+) Intraepithelial Lymphocyte (IEL) population is present, and 50-60% of these patients develop Enteropathy-Associated T-cell lymphoma (EATL). TCRγδ+ IELs play an important role in mucosal repair, homeostasis, and tumor surveillance. Recently, human small intestinal TCRγδ+ IELs were shown to have regulatory potential in uncomplicated coeliac disease (CD).
Aim: In the present study we investigated whether TCRγδ+ IELs are decreased in RCD II, providing a possible explanation for persisting mucosal damage and inflammation, and the emergence of aberrant T-cells with clonal expansion to EATL.
Design and Methods: Multiparameter flow cytometric immunophenotyping was performed on IELs isolated from fresh small bowel biopsy specimens of relatively large distinct CD patient and control groups (n=87).
Results: A significantly lower percentage of TCRγδ+ IELs was found in RCD II as compared to all other CD groups. In contrast, in uncomplicated CD patients significantly more TCRγδ+ IELs were found than in controls. Overall, there is a clear negative relation between TCRγδ+ IELs and aberrant IELs. Interestingly, TCRγδ+ IELs increase again in RCD II after effective therapy.
Conclusions: The observed negative relation between TCRγδ+ and aberrant IELs, along with their known regulatory capacity in uncomplicated CD, implies that TCRγδ+ IELs may play a crucial role in mucosal repair, regaining homeostasis and possibly even tumor surveillance. These cells may be important markers, in addition to the aberrant T-cells, to differentiate between disease categories and to evaluate the effectiveness of therapeutic strategies.

Introduction

T-cell receptor (TCR)γδ+ T cells are only a small fraction of circulating lymphocytes (2-5%, the Vδ2 subset), but can be found in high numbers in epithelial surfaces (the Vδ1 subset). In the intestinal tract, they can comprise up to 50% of the residing T-cell population.[1] These TCRγδ+ Intraepithelial Lymphocytes (IELs) should be regarded as a separate lymphocyte lineage from TCRαβ+ IELs because of different development and the ability to recognize soluble antigens without restriction by polymorphic MHC class I or class II molecules.[2] Over the years, increasing evidence revealed that these cells play a unique role in maintaining the integrity of the intestinal mucosa in response to tissue injury due to infection, inflammation, or malignancy. [3-6] However, the exact role of these TCRγδ+ IELs in humans was not fully understood until recently.
Coeliac disease (CD) is a T-cell mediated disease of the small intestine triggered by the ingestion of dietary wheat gluten in genetically predisposed individuals.[7;8] It leads to malabsorption due to mucosal damage (villous atrophy, crypthyperplasia, and intraepithelial lymphocytosis[9]) and commits the patient to a life-long gluten-free diet (GFD), which is sufficient to treat the overwhelming majority of patients. However, in

a small percentage (2-5%) of adult onset CD serious complications develop in the form of refractoriness and the development of (pre-)malignant complications. Patients with CD are defined as suffering from refractory coeliac disease (RCD) when clinical and histological symptoms persist or recur, after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 months. [10-13] IELs with an aberrant immunophenotype are known to be a prognostic parameter in RCD and their presence is associated with the development of Enteropathy Associated T-cell Lymphoma (EATL).[11;14;15] Cellier et al. have first shown that RCD is associated with this abnormal subset of IELs, expressing cytoplasmic CD3ε and restricted rearrangements of the TCRγ chain, but lacking surface expression of T-cell markers CD3, CD4 and CD8.[16] When normal expression of T-cell surface markers occurs (RCD I), the prognosis is less dismal than when an aberrant IEL population is present (RCD II); 50-60% of the latter patients develops EATL within 4-6 years.[15] These EATLs are thought to arise from the IEL compartment, displaying a phenotype comparable to the aberrant IELs in RCD II.[17]

In recent years, research on RCD has focussed on the innate response in the intraepithelial layer with emphasis on the NK-like properties of TCRαβ+ IELs and their response to gluten, activating a cascade with high levels of IL-15.[18-20] IL-15 levels correlated with the degree of mucosal damage and the highest levels were found in complicated CD patients with EATL,[21] possibly perpetuating epithelial damage and promoting the emergence of an aberrant T-cell population. In contrast, IL-15 levels significantly decrease in uncomplicated CD patients on a GFD compared with active CD [20], in parallel with a decrease in TCRαβ+ IELs.[22;23] However, the TCRγδ+ IELs in uncomplicated CD remain elevated several years after initiation of a GFD.[22;24;25] Their exact role remained elusive for many years, but recently Bhagat et al.[26] have described the regulatory/suppressor function of human small intestinal TCRγδ+ IELs. TCRγδ+ IELs from uncomplicated CD patients had the potential to suppress the IL-15 induced cytotoxic phenotype of CD8+TCRαβ+ IELs in vitro by secreting transforming growth factor beta (TGF-β).[26]

To our knowledge, the present study is the first report on the dynamics of TCRγδ+ IELs in RCD, and their relationship with aberrant IELs. In the current report, we investigated relative levels of TCRγδ+ IELs in the intestinal mucosa of RCD patients in comparison to other CD disease and control groups. Our results show that TCRγδ+ IELs are decreased in RCD II, possibly contributing to loss of homeostasis, impaired mucosal repair and the emergence of aberrant T-cells, ultimately progressing to EATL.

Patients, material and methods

Patients

Eighty-seven flow cytometric analyses on 3-4 small intestinal spike biopsy specimens were performed in consecutive subjects evaluated for RCD and CD between January 2006 and July 2007. The included patients were subdivided into 4 subgroups:

- Controls without CD, (n=21, 7M/14F, mean age 40 years, SD 15 years, range 18-69 years). In this group, CD was excluded by small intestinal biopsy. Biopsy specimens were collected based on symptoms that varied from aphtous stomatitis, reflux,

nausea and dyspepsia to diarrhoea, weight loss, abdominal pain, and osteopenia. Some patients had a (first-degree) relative suffering from CD, and were evaluated to exclude CD as a potential cause of their symptoms. None of these controls were on a GFD. Besides normal small intestinal histology, all patients showed normal serology with respect to CD –associated antibodies.

II Untreated CD (n=9, 3M/6F, mean age 50 years, SD 11 years, range 35 - 68 years). Patients in this group showed signs of malabsorption, positive CD serology (antibodies against endomysium (EMA) and tissue transglutaminase (tTGA)), as well as typical CD histopathology (villous atrophy, crypthyperplasia, and intraepithelial lymphocytosis). These patients were not on a GFD yet.

III Treated CD patients (n=27, 7M/20F, mean age 57 years, SD 12 years, range 30-77 years). This group consisted of biopsy proven CD patients, with a documented histological and serological response to gluten withdrawal, on average 47 months on a GFD (range 10-181 months, SD 50).[12]

IV Patients with RCD (total n=30), considered to be refractory when symptoms of malabsorption due to villous atrophy persisted or recurred after a former good response on a GFD. Signs and symptoms were comparable to those described in previous studies.[15] Histopathology of these patients showed at least partial villous atrophy (Marsh IIIA) and other causes of villous atrophy had been excluded, including Whipple's disease, Crohn's disease, tuberculosis, radiation enteritis, AIDS, common variable immunodeficiency syndrome, eosinophilic gastroenteritis, auto-immune enteropathy and immunoproliferative small intestinal disease, giardiasis, postinfectious diarrhea, tropical sprue, collagenous sprue, and protein intolerance.[12;13] Furthermore, absence of both HLA-DQ2 and -DQ8 haplotypes virtually ruled out RCD, given its high negative predictive value for (R)CD.[27]

Considering the fact that a significant number of patients (around 50%) suspected for RCD may indeed experience inadvertent gluten ingestion [28], their dietary compliance was carefully evaluated by a dietician, and confirmed by negative CD serology. In the RCD patient-group the presence of an EATL was excluded by radiological and endoscopic methods, including small intestinal follow- through, computed tomography scanning of thorax and abdomen,[29] whole body positron emission tomography scan, [30] upper gastrointestinal endoscopy, video capsule endoscopy and/or double balloon enteroscopy [31], as well as trephine bone marrow biopsy specimens.

Based on clinical presentation and flow cytometric analysis, the RCD group was subdivided into type I and II using the 20% cut-off value for aberrant IELs (surface CD3- CD4/8- CD7+ cytoplasmic CD3+), as established previously.[14;15] This cut-off value has proven to be reliable for early risk stratification [15] and targeted therapeutic options in RCD patients.[32-34] The total group consisted of 14 RCD I patients (2M/12F, mean age 60 years, SD 10 years, range 38 - 76 years) and 16 RCD II patients (7M/9F, mean age 63 years, SD 8 years, range 46 - 78 years).

In five RCD II patients treated with Autologous Stem Cell Transplantation (ASCT) or Cladribine (2-CDA) [32;33], the percentages TCRγδ+ and aberrant IELs were evaluated before treatment and after 3 - 6 - 12 - 24 - 36 months after therapy.

Isolation of IELs from small intestinal biopsy specimens and flow cytometry

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe®) were taken from the second part of the duodenum.[12] For flow cytometric evaluation 3-4 biopsy specimens were taken and immediately analyzed. All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.

Intraepithelial lymphocytes were isolated from intestinal biopsies as originally described by Madrigal et al.[35] with minor modifications. Briefly, biopsies were vigorously shaken at 37˚C for 60 min in phosphate buffer solution (PBS) supplemented with 1mM DTT (Fluka BioChemika, Buchs Switzerland) and 1mM EDTA (Merck, Darmstadt, Germany). The released IELs were washed twice with PBS supplemented with 0,1% BSA (bovine serum albumin) (Roche Diagnostics, Mannheim Germany) and subsequently stained for 30 minutes on ice, with fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, CD7, CD45, γδTCR (all from from BD Biosciences, San Jose CA). Cytoplasmic staining of CD3 was performed after cell permeabilization (Cytofix/CytoPerm Plus™ kit by BD Biosciences). Flow cytometric analysis was performed on a standard 4-color Fluorescence- Activated Cell Scanner (FACSCalibur, BD Biosciences). The data were analyzed using Cellquest software (BD Biosciences). Care was taken to analyze only viable cellular events based on light scatter properties. All analyses were performed on lymphocytes, based on bright CD45 staining and low sideward scatter. Aberrant T cells were defined as CD7+ cytoplasmic $CD3^{+}$, surface CD3, CD4 and CD8 negative cells, as described previously.[14]

Histopathological findings on sections of formaline-fixed biopsy specimens were classified using the modified Marsh criteria for the gluten-sensitive spectrum.[9;36;37]

Statistical analysis

Analysis of variance (ANOVA) of the data, for comparison between the groups, was performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL). A Bonferroni correction for multiple testing was performed. To determine the relation between the percentages aberrant and TCRγδ+ IELs, the 2 RCD groups were joined together, because the percentage aberrant IELs are the main criterium on which the groups are separated. RCD was compared to untreated CD and CD on a GFD. A value of $p<0.05$ was considered statistically significant.

Results

Analysis of TCRγδ+ IELs in CD and in RCD

Figure 1 shows box- and whisker plots of the log-transformed percentages of TCRγδ+ IELs in the different groups analyzed. ANOVA revealed a significant difference in the relative amount of TCRγδ+ IELs (percentage of total IEL) among the groups ($p<0.0001$). Bonferroni correction showed that in CD patients, both untreated and those on a GFD, significantly more TCRγδ+ IELs were found than in controls without CD ($p=0.001$ and $p=0.012$, respectively). In contrast, there are significantly less TCRγδ+ IELs in RCD II as compared to all other CD groups (vs. untreated CD: $p< 0.0005$; vs. CD on GFD: $p<0.0005$; vs. RCD I: $p=0.001$).

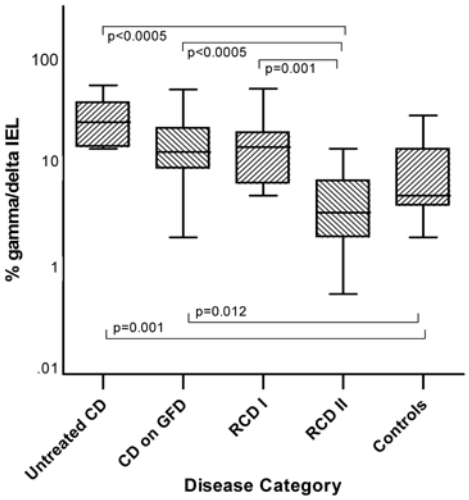

Figure 1. The log-transformed percentage TCRγδ+ IELs in duodenal biopsy specimens in each disease category. In RCD II significantly less TCRγδ+ IELs were found than in all other groups, with exception of the controls. Untreated CD p<0.0005, CD on GFD p<0.0005, RCD I p= 0.001. Furthermore there were significantly more TCRγδ+ IELs in CD, both untreated (p=0.001) as on a GFD (p=0.012), as compared to controls without CD.

The relation between TCRγδ+ and aberrant IELs

In order to investigate the relation between TCRγδ+ and aberrant IELs, their levels were directly compared with the different CD patient groups. **Figure 2** shows the direct relation between both IEL subsets. Statistical analysis of the percentages aberrant IELs in the CD patient groups compared with the percentages TCRγδ+ IELs revealed a significant negative relation (p=0.001). The negative relation found primarily results from the presence of aberrant IELs in conjunction with the lack of TCRγδ+ IELs in RCD II, as opposed to the opposite in all other CD groups.

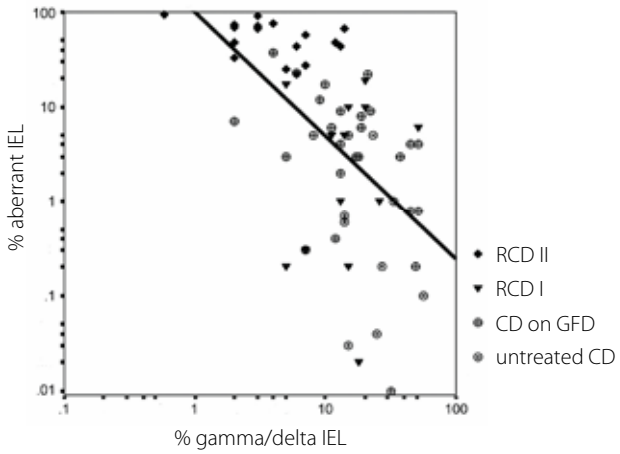

Figure 2. The relation between the percentage TCRγδ+ IELs and the percentage aberrant IELs. Analysis of variance on the percentage aberrant T-cells, revealed that there is a significant relation with the percentage TCRγδ+ IELs (p=0.001).

Analysis of TCRγδ+ IELs in RCD II patients after successful therapy

The upper part of **table 1** displays the course of the percentage TCRγδ+ IELs in three RCD II patients, which had been successfully treated with ASCT or Cladribine.[32;33] The follow-up of these patients was 20, 39 and 18 months, respectively; all patients are doing clinically well and none of them has developed EATL as yet. Upon recovery of mucosal villous atrophy and a concomitant decrease in aberrant IELs, below 20%, the percentage TCRγδ+ IELs increases markedly in all cases. The latter is illustrated by a decrease of the aberrant IEL / TCRγδ+ IEL ratio to a value <1.0.

Age, gender and treatment	Marsh category		CD7+ cyt CD3+surf CD3- % of IEL		TCRγδ+ % of IEL		Ratio aberrant IEL / TCRγδ+ IEL	
Patient	Before	After	Before	After	Before	After	Before	After
		3 – 6 – 12 – 24 –36 – 42 months		3 – 6 – 12 – 24 –36 – 42 months		3 – 6 – 12 – 24 –36 – 42 months		3 – 6 – 12 – 24 –36 – 42 months
F68 yr, 2-CDA	IIIA	II - II - I	57	4 - 1 - 11	7	14 - 17 - 17	8.1	0.3 - 0.1 - 0.6
M68 yr, ASCT	IIIB	I - X - I - I - 0	51	2 - X - 6 - 4 - 12	11	19 - X - 33 - 45 - 37	4.6	0.1 - X - 0.2 - 0.1 - 0.3
F67 yr, 2-CDA	IIIC	IIIB –IIIA – I	71	60 – 14 – 16	5	7 – 21 – 25	14.2	8.6 – 0.7 – 0.6
M66 yr, ASCT	IIIA	IIIA – X – IIIA – I – IIIA/EATL	94	89 – X – 86 – 25 – 87 – 64	1	2 – X – 2 – 5 – 2 – 6	94.0	44.5 – X – 43.0 – 5.0 – 43.5 – 10.7
F64 yr, ASCT	IIIC	IIIA – X – IIIA – II	59	34 – X – 27 – 66	5	8 – X – 12 – 4	11.8	4.2 – X – 2.2 – 16.5

Table1. The dynamics of TCRγδ+ IELs and aberrant IELs (as percentage of total IELs) before and after treatment in RCD II patients. Histological and phenotypical flow cytometric analyses of IELs in duodenal biopsies of five RCD II patients before (1-6 months) and after treatment with Autologous Stem Cell Transplantation or Cladribine therapy. The upper part shows three patients after successful treatment, follow up is respectively 21, 40 and 19 mo., all these patients are doing clinically well and none of them has developed EATL thus far. The lower part depicts two patients in whom therapy was less successful, M66 developed EATL at 33 mo. follow-up and deceased at 44 mo. F64 is still alive at 31 mo. follow-up.

Normal range for CD7+cytCD3^{+}surfCD3^{-} percentage of IEL < 20%. T cell receptor γ–gene rearrangement was monoclonal in all five patients. X = no follow-up data available.

Interestingly, no increase in TCRγδ+ IELs was observed when therapy did not succeed to mediate a decrease in the percentage aberrant IELs and mucosal recovery. Two patients in whom therapy was not successful are shown at the lower part of **table 1**. The male patient who was 66 years old at the time of ASCT developed EATL 33 months after transplantation and deceased at 44 months follow-up. The female patient is doing clinically well at 31-months follow-up, but has persisting aberrant IELs and the mucosa is still not fully recovered. Both patients had persisting high ratios aberrant IEL TCRγδ+ IEL, never below 1.

Discussion

Previous studies have emphasized the possible role of intestinal IELs in the pathogenesis of RCD. In our previous work we showed that quantification of aberrant IELs by flow cytometry is well suited for the identification and follow-up of those RCD patients (type II) at risk for EATL.[14;15] The underlying mechanism by which aberrant IELs, which are cytologically normal and do not form tumor masses, eventually evolve to overt EATL in the majority of patients (50-60% within 4-6 years[15]) remains to be established. However, a central role for IL-15 has been proposed, as this is overexpressed in RCD II and EATL.[18;20;21] TCRγδ+ IELs are thought to represent an important regulatory T-cell subset and play a key role in maintaining mucosal homeostasis and regulating inflammatory responses (reviewed by Nanno et al.[38]). Recently, the first report on the function of human intestinal TCRγδ+ IELs [26] provided evidence for the regulatory capacity of TCRγδ+ IELs, found previously in mouse studies, and their ability to downregulate TCRαβ+ IEL-mediated immune responses.[1;3-6;39] Other mouse studies have suggested TCRγδ+ IELs to function also as part of the innate immune surveillance of tumors.[40] For example, TCRγδ+ IELs had the capacity to inhibit cutaneous tumor development.[41] Furthermore, a higher level of IL-15, which inhibits activation-induced cell death and promotes lymphomagenesis, was expressed by the intestinal epithelial cells of TCRγδ+ IEL-deficient mice.[6] In the present study, we investigated to what extent the TCRγδ+ IEL population is affected in RCD II, thereby possibly contributing to loss of homeostasis, impaired mucosal repair, and the emergence of the aberrant T-cells, ultimately progressing to EATL.

We found a significantly lower proportion of TCRγδ+ IELs in RCD II as compared to all other CD groups. In contrast, significantly higher percentages of TCRγδ+ IELs were found in coeliac disease patients, both untreated and responding to a GFD, as compared to controls without CD. This is in agreement with literature indicating CD to be characterized by a permanent increase of TCRγδ+ IELs with a concomitant elevation of infiltrating TCRαβ+ IELs during the active stage of the process.[22;24;25] However, the TCRαβ+ IELs decrease within months in response to gluten withdrawal, whereas for TCRγδ+ IELs this may take years to occur.[22;23] The contribution of TCRγδ+ IELs to the pathogenesis of villous atrophy in CD still remains unclear. Their persistent increase in CD patients whose mucosa has recovered upon a GFD suggests that TCRγδ+ IELs do not induce the epithelial damage directly. This is supported by several studies that

showed a greater number of TCRγδ+ IELs in latent CD and dermatitis herpetiformis, in which the mucosa was still unaffected but progressed to villous atrophy eventually.[42;43] In contrast, the presence of TCRαβ+ IELs correlates directly with the degree of villous atrophy in CD patients.[22] All together, TCRγδ+ IELs may be important in initiating the early response in CD, preceding mucosal damage, promoting the development of TCRαβ+ IEL expansion. During gluten withdrawal and subsequent contraction of the TCRαβ+ IEL response, however, TCRγδ+ IELs may take on an anti-inflammatory role initiating mucosal repair.[2] Evidence for this has recently been provided by Bhagat et al.[26] who have shown that the human TCRγδ+ IELs with the most potent regulatory potential increase upon commencement of a GFD in uncomplicated CD. As compared to active CD, TCRγδ+ IELs in treated CD showed greater capacity to suppress the cytotoxic arming of CD8+TCRαβ+ IELs via TGF-β production.

It still remains to be investigated whether the relative deficiency in TCRγδ+ IELs in RCD II is the cause or consequence of the expansion of the aberrant IEL population in RCD II. The scarcity of TCRγδ+ IELs and the resulting possible shortage of sufficient TGF-β production may result in persisting epithelial damage and high IL-15 production, leading to a cascade of ongoing expansion aberrant IELs with IFN-γ production and cytotoxicity against enterocytes. Alternatively, Bhagat et al.[26] speculated that due to excessive amount of IL-15 that continually stimulates TCRαβ+ IELs in active CD, the suppressive abilities of TCRγδ+ IELs may be overwhelmed until gluten is withdrawn from the diet and IL-15 levels diminish.[26] In RCD II, however, the continuous high levels of IL-15 despite the GFD [21] may result in greater survival and/or selective proliferative advantage of aberrant IELs. Indeed, the concentration of IL-15 that is required to induce growth and promote survival of aberrant clonal IELs has been shown to be much lower than that required to induce growth in the normal intraepithelial T-cell population.[20] This possibly results in a progressive relative disappearance of normal IELs, specifically TCRγδ+ IELs, while expansion of aberrant IEL may be facilitated, ultimately resulting in lymphomagenesis.

Interestingly, our results show that TCRγδ+ IELs can increase again upon therapy directed at the elimination of aberrant IELs in RCD II. Autologous stem cell transplantation or chemotherapy may mediate interruption of the above-mentioned cascade, allowing recovery of the TCRγδ+ IEL population and revival of homeostasis. The latter is illustrated by a decrease of the aberrant IEL / TCRγδ+ IEL ratio to a value <1.0.

Translating these results from 'bench to bedside', the relative absence of TCRγδ-IELs confirms the presence of aberrant IELs in RCD. Given the high risk of lymphoma development in RCD II and the resulting impact on survival and therapeutic options, these cells may be imporant markers of disease progression. The combination of these two markers improves on the specific and sensitive identification of RCD II patients. Since diagnostic flow cytometry facilities are currently available in most clinical centres, analysis of aberrant IEL and TCRγδ+ IEL in complicated CD should be as accessible as analysis of CD4+ T-cells in HIV-patients. In comparison, immunohistochemistry is more demanding in accurate quantification of lymphocyte subsets in biopsy specimens as compared to flow cytometry.

In conclusion, the current study indicates the potential involvement of TCRγδ+ IELs in the pathogenesis of RCD II and EATL development. In addition to the recently published functional evidence on the regulatory potential of human small intestinal TCRγδ+ IELs[26], we propose TCRγδ+ IELs to be an important additional marker to differentiate between CD disease categories and to evaluate the effectiveness of therapeutic strategies in RCD II. T-cell flow cytometry appears mandatory in the work-up and follow-up of complicated CD. The current paper provides a novel marker in this approach.

Study highlights

What is current knowledge

- Refractory Coeliac Disease, with persisting mucosal damage, can be subdivided into two groups based on the presence (RCD II) or absence (RCD I) of intraepithelial lymphocytes (IELs) with an aberrant expression of T-cell surface markers.
- In contrast to RCD I, the prognosis of RCD II is dismal; 50-60% of the latter patients develops Enteropathy Associated T-cell Lymphoma within 4-6 years.
- Recently, human small intestinal TCRγδ+ IELs were shown to have regulatory potential in uncomplicated coeliac disease, in which high numbers of these cells are found.

What is new here

- The current study indicates the potential involvement of TCRγδ+ IELs in the pathogenesis of RCD and lymphoma development.
- The proportion TCRγδ+ IELs was found to be significantly decreased in RCD II, but increased again after effective therapy upon elimination of the aberrant IELs.
- The combination of the two markers improves on the specific and sensitive identification of RCD II patients, as well as on the evaluation of the effectiveness of therapeutic strategies**.**
- T-cell flow cytometry appears mandatory in the work-up and follow-up of complicated CD, and this article provides a novel marker in this approach.

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8.
Decreased numbers of circulating iNKT cells in Refractory Coeliac Disease

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Abstract

Introduction: A small proportion of coeliac disease (CD) patients become refractory (RCD) to a gluten-free diet (GFD) showing uncontrolled immune mediated enteropathy. Some of these patients exhibit a high risk to develop enteropathy-associated T-cell lymphoma (EATL).
Aim: The aim of the study was to find whether a lack of circulating homeostatic T cells, such as Treg, Tγδ+ or iNKT cells would be associated with the development of RCD or EATL.
Patients and methods: We investigated in a total of 137 CD patients (28 untreated, 80 responsive to GFD and 29 RCD (14 type I and 15 type II)) and 73 age-matched healthy volunteers the frequency of Treg, Tγδ+ and iNKT lymphocytes by flow cytometric analysis of peripheral blood.
Results: Circulating Tγδ+ cell and Treg frequencies in RCD were comparable to those in healthy controls. However, RCD patients had significantly reduced numbers of circulating iNKT cells, as compared to age-matched patients responding to a GFD. This reduction was similar in RCD patients with and without aberrant intraepithelial lymphocytes and in patients with EATL. Circulating iNKT cell numbers were not reduced in untreated coeliac patients. GFD treated patients had a significantly increased proportion of CD4+ iNKT cells.
Conclusion: Follow-up studies are necessary to determine whether CD4+ iNKT cells control the immune response against gluten and their abscence contributes to the progression to RCD and EATL.

Introduction

Coeliac Disease (CD) is an immune-mediated enteropathy caused by the ingestion of wheat and other gluten-containing cereals (rye, barley and probably oats) in genetically predisposed individuals[1] leading to intestinal villous atrophy. This is characterized by crypt hyperplasia and increase of intraepithelial lymphocytes (IELs). The current treatment is a life-long strict gluten-free diet (GFD) resulting in a complete remission of the symptoms and returning to a normal small intestinal mucosa. However, a small proportion of CD patients become unresponsive to a GFD, a stage known as refractory CD (RCD) [2]. This is characterized by an uncontrolled, gluten independent, intestinal immune reaction. As a result, the IEL of the diseased epithelium can continue to undergo NK-like reprogramming [3] with T cell receptor (TCR)-independent IFN-γ production and cytotoxicity. Additionally, an aberrant IEL population, lacking surface expression of CD3 and CD8, appears in a subgroup of RCD patients (type II) which have a high risk to develop an enteropathy-associated T-cell lymphoma (EATL), whereas RCD type I patients show normal expression of T-cell antigens and have a better prognosis [2, 4].
CD4+ CD25+ regulatory T cells (Tregs; CD3+, CD4+, CD25+ and intracellular transcription factor Forkhead Box P3+ (FoxP3+)), invariant NKT cells (iNKT; CD3+, TCR Vα24+Vβ11+)

and to a lesser extent TCR γδ+ lymphocytes (Tγδ; CD3+, TCR γδ+) are lymphocyte populations that help to maintain immune homeostasis [5-10]. Tregs [11] elicit their function by suppressing IL-2 production and T-cell proliferation [12, 13]. Intraepithelial Tγδ, appear to play a key role in oral tolerance by inducing T regs [14, 9]. After TCR-triggering, Tγδ cells rapidly but transiently express the lymph node-homing receptor CCR7. Once in the lymph nodes, they may act as professional antigen presenting cells inducing proliferation and differentiation of naïve T cells [15].

Human iNKT cells express classical NK cell markers as well as an invariant T cell receptor (TCR Vα24+Vβ11+), which recognizes antigens presented by the MHC class I-like molecule CD1d [13, 16-19]. iNKT cells can also be sub-divided in CD4+ and CD4- (most of these CD4-CD8-) cells. CD4-CD8- iNKT cells predominantly produce TH1 cytokines (IFNγ and TNFα) whereas CD4+ iNKT cells can produce both TH1 and TH2 (IL4 and IL13) cytokines [20, 8]. Because of their unique capacity to rapidly produce large quantities of both TH1 (IFNγ) and TH2 (IL4) cytokines upon stimulation [21, 22], iNKT probably play a key role either in the protection against tumors or in preventing autoimmune disease [18, 23, 24].

Although circulating iNKT, Tγδ and Treg cell numbers are relatively low (approximately 0.02-0.2% of the CD3+ T-cells are iNKT cells (24) 2-10% of the CD3+ cells are Tγδ cells [15] and 2-5% of CD4+ T-cells are Tregs [11]), numeric deficiencies have been reported in autoimmune disease and in malignancy [18, 23, 25, 26, 27]. Reduced circulating numbers of both iNKT cells and Tγδ+ cells have been reported in CD [28, 29]. However, these studies have not been performed in relation to the development of uncontrolled autoimmunity as seen in RCD or to the development of malignancies as EATL. In addition, since iNKT can be either regulatory or pro- inflammatory, expression of the regulation-associated proteins FoxP3 and CTLA4 in iNKT cells might provide new information on the actual regulatory function.

The hypothesis proposed in this study is that a lack of regulatory T cells, including CD4+ iNKT cells, Treg and Tγδ cells, predisposes to the development of RCD. To this end, circulating levels of these cells were assayed in a group of RCD patients (both type I and type II), as well as in active (untreated) and GFD-treated CD patients and in age-matched healthy controls. Moreover, the intracellular levels of the regulatory proteins CTLA4 and FoxP3 were determined in iNKT cells.

Materials and methods

We studied a total of 137 CD patients, 28 untreated (mean age 26.5 years; range 1-75 years, 36% male), 80 responsive to GFD (mean age 38.2 years; range 3-76 years 22% male) and 29 refractory CD (RCD) not responding to a GFD (mean age 57.50 years; range 45-68 years 38% males) and 73 age-matched healthy volunteers without known autoimmune diseases or malignancies (mean age 32.2 years; range 2-82 years; 36% male) and RCD type II (15 patients, mean age: 60.6 years; range 45-68 years; 39% male) according to the absence or presence (>20% of IEL) of aberrant intraepithelial lymphocytes respectively [30]. At the time of diagnosis all CD patients,

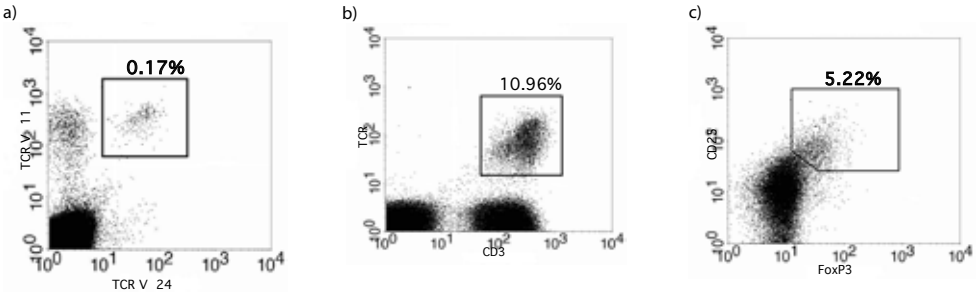

Figure 1. Analysis of circulating regulatory cells from one representative donor. Live lymphocytes were gated based on forward and sideward scatter.

(**a**) iNKT cell analysis. Example of staining of PBMCs for Vα24 and Vβ11 staining on gated CD3+ lymphocytes. iNKTs were defined as CD3+ Vα24+ and Vβ11+

(**b**) TCR Tγδ+ analysis. Example of staining of PBMCs for Tγδ and CD3 expression on gated live lymphocytes. TCR Tγδ+ cells were defined as CD3+ Tγδ+.

(**c**) Treg analysis. Example of staining of gated CD3+CD4+ T cells for surface CD25 and intracellular and FoxP3 expression. Treg cells were defined as surface CD3+ CD4+ CD25high and intracellular FoxP3+.

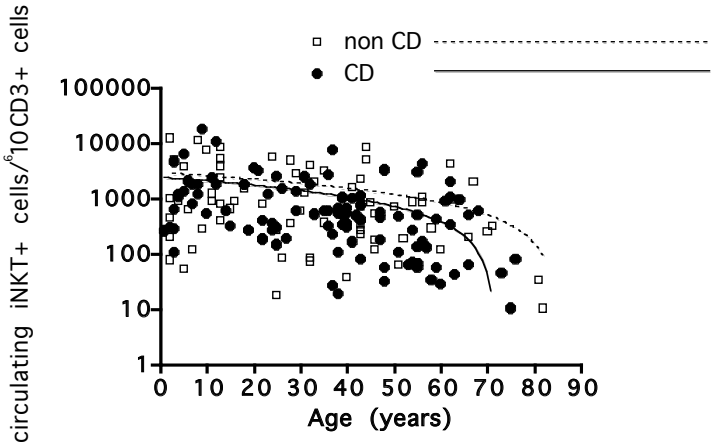

Figure 2. Effect of age on circulating iNKT cell frequencies in healthy controls without known autoimmune diseases or malignancies (non CD) and Coeliac Disease (CD) patients. Scatter diagrams with regression lines show an age-dependent reduction in circulating Va24αVb11β iNKT cells/10^6 T-cells in controls (Spearman´s r= -0.238, p=0.0430) and CD patients (Spearman´s r= -0.4104, p<0.0001).

responding and non-responding, had positive endomysium antibodies, carried the HLA-DQ2 and/or DQ8 as well as typical mucosal changes in the duodenal biopsy. Responding GFD-CD were characterized by a clinical, serological and pathological recovery upon GFD while non-responding patients (RCD) only had serological recovery upon the GFD. At the time of performing the blood test for this study, active CD patients had positive serology and villous atrophy but all responding GFD-CD and non-responding RCD patients had negative serology for at least 1 year. The study was approved by the medical ethical committee of the VU university medical centre, Amsterdam.

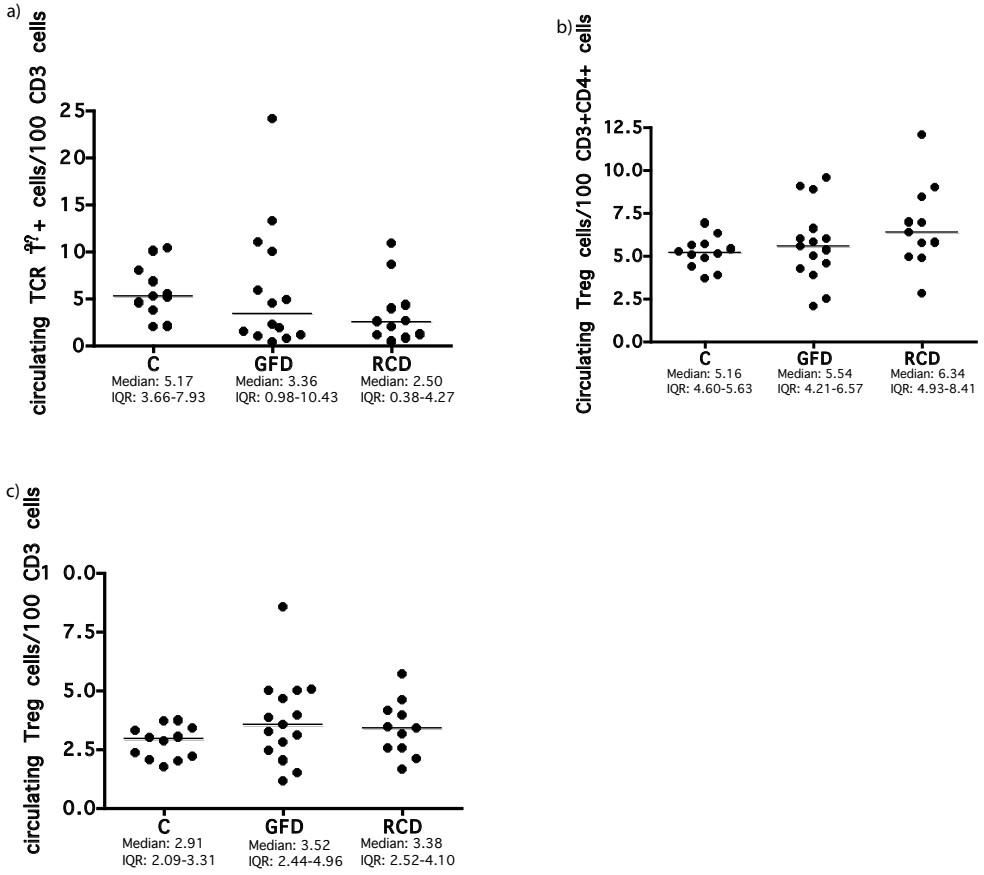

Figure 3. Percentages of circulating Tγδ+ and Tregs in peripheral blood of healthy controls without known autoimmune diseases or malignancies (C), GFD responding (GFD) and GFD non-responding (RCD) CD patients. **a**) Tγδ+ cells within CD3+ T cell population, **b**) Tregs within CD3+CD4+ T cell population and **c**) within the CD3+ population. No statistically significant differences were observed (Krustall-Wallis test, p=0.1910, p=0.1804 and p=0.2185 respectively). Horizontal bars indicate median values. IQR: interquartile range, n: sample size.

Flow cytometric analysis of peripheral blood

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-hypaque density gradient centrifugation and the circulating cell numbers of the regulatory cell subsets were determined by flow cytometry on a FACS Calibur (BD Biosciences). iNKT cells were identified by coexpression of CD3 (APC-labelled, BD Biosciences), Vα24 (FITC-labelled, Immunotech) and Vβ11 (PE-labelled, Immunotech). Intracellular levels of CTLA4 (APC-labelled, BD Biosciences) and FoxP3 (APC-labelled, eBioscience) were also determined in iNKT cells combined with surface expression of CD4 (PerCP-labelled, BD Biosciences). Tregs were identified by coexpression of CD3 (PerCP-labelled, BD Biosciences), CD4 (FITC-labelled) and high expression of CD25 (APC-labelled, BD Biosciences) as well as intracellular FoxP3 (PE-labelled, eBioscience). Intracellular staining was done according to the manufacturer's instructions. Tγδ cells were identified by coexpression of CD3 (PerCP-labelled) and γδ TCR (PE-labelled). Appropriate isotype controls were used in all experiments.

A minimum of 100,000 viable lymphocytes were acquired per patient for Treg, Tγδ and iNKT cell determination.

Statistical analysis

Correlation analyses and two-tailed non-parametric statistical analyses were performed using the Kruskal-Wallis one-way analysis of variance test and the Mann-Whitney U test. $P<0.05$ was considered significant.

Results

Phenotypical analysis of regulatory T-cells subsets

Examples of phenotypical analysis of circulating iNKTs, Tregs and Tγδ cells by flow cytometry are shown in **figure 1**. Live lymphocytes were gated based on forward sideward scatter. Unless otherwise stated, iNKT, Treg and Tγδ cell frequencies are given as fraction of the CD3+ population.

Since it is known that some regulatory T cell subset frequencies decrease with age[31] and may be influenced by gender, linear regression analysis was performed. No gender effect was observed for any subset, while an age related decline was observed for iNKT cell frequencies both in healthy controls (Spearman´s $r= -0.2376$, $p=0.0430$) and CD patients (Spearman´s $r= -0.4104$, $p<0.0001$; **figure 2**)

Circulating Tγδ and Treg cell numbers are normal in CD and RCD

No significant differences in circulating Tγδ cell frequencies between responding CD, RCD and healthy controls were observed (**Figure 3a**). Circulating levels of CD4+CD25+FoxP3+ Tregs were determined both within the CD3+CD4+ (**Figure 3b**) subset and the total CD3+ (**Figure 3c**) population. No differences in circulating Treg frequencies were observed in any case. Finally, within the RCD group, there was no correlation between the circulating levels of either Treg and Tγδ cells and the percentage of aberrant IELs (data not shown).

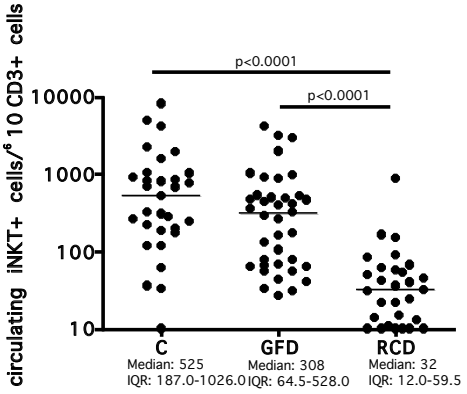

Figure 4. Circulating iNKT numbers per million of circulating CD3+ cells in peripheral blood of GFD non responding (RCD) and responding (GFD) CD patients and healthy controls without known autoimmune diseases or malignancies (C) (over 40 years). Statistically significant differences are shown (two-tailed Mann-Whitney U test; Krustall-Wallis test p<0.0001). Horizontal bars indicate median values. IQR: interquartile range, n: sample size.

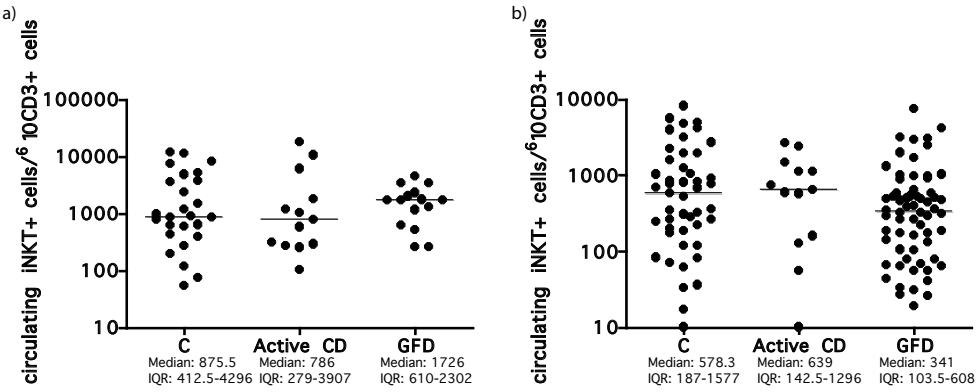

Figure 5. No relation between iNKT cell frequency and aberrant intraepithelial cells or the development of enteropathy-associated T-cell lymphoma (EATL) in Refractory Coeliac Disease (RCD). **a**) iNKT cell numbers frequency related to the percentage of aberrant (surface CD3-, intracellular CD3+) intraepithelial cells in RCD patients (Spearman´s r= 0.2177, p=0.3303) and **b**) comparison of circulating iNKT cell frequency among RCD patients with or without EATL. (two-tailed Mann-Whitney U test, p=0.6588). Horizontal bars indicate median values. IQR: interquartile range, n: sample size.

iNKT cell numbers are selectively decreased in RCD

iNKT cell frequencies in RCD patients, which were generally older than the control group (mean age 57.5 years, range 45-68 years), were compared to iNKT cell frequencies in a group of age-matched responding CD patients (38 individuals, mean age 55.3 years, range 41-76 years) and healthy controls (31 individuals, mean age 53.8 years, range 41-82 years) to correct for the age-related effect on circulating iNKT cell numbers. **Figure 4** shows that the iNKT cell population was reduced in RCD patients (median 32.0) compared to responding CD patients ($p<0.0001$, median 308.0) and healthy controls ($p<0.0001$, median 525.0)

Reduction in iNKT cell numbers is not related to the presence of aberrant IEL or EATL in RCD

To investigate whether low iNKT cell numbers in RCD would be associated with the presence of potentially pre-malignant, aberrant IEL (surface CD3-, intracellular CD3+), we compared iNKT cell numbers with the percentage of aberrant IEL in RCD patients (**Figure 5a**). No significant correlation was found and consequently no difference was found in the circulating numbers of iNKT cells between RCD I and RCD II (RCD I median: 33.5, IQR 10.5-59.5; RCD II median: 32.0, IQR 13.0-67.0). Moreover, no further circulating iNKT cell depletion occurred in the 7 RCD patients that developed an EATL (**Figure 5b**).

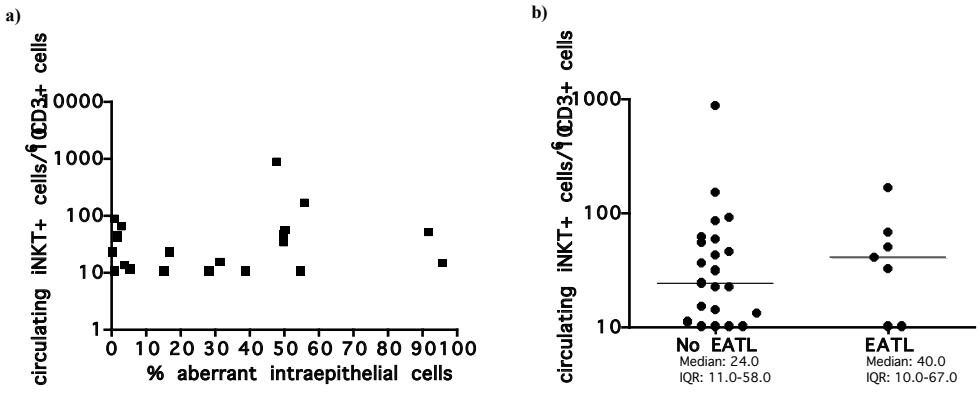

Figure 6. Frequency of circulating iNKT cells (number of iNKT cells per million of circulating CD3+ T cells) in peripheral blood of healthy controls without known autoimmune diseases or malignancies (C), untreated patients with active CD (active CD) and GFD responding patients (GFD). **a**) children below 20 years, and **b**) adults over 20 years. No statistically significant differences were found (Krustall-Wallis test, p=0.7008 and p=0.1467 respectively). Horizontal bars indicate median values. IQR: interquartile range, n: sample size.

Circulating iNKT cell numbers are normal in untreated or treated CD

We then checked in how far the numeric defect in NKT cells was characteristic for the refractory state of coeliac patients or whether patients with active coeliac disease before treatment would already have reduced iNKT cell numbers, as was recently reported by Grose *et al* [28]. Separate evaluations were performed for children (below 20 years) and adults (over 20 years), after confirming that there was no iNKT depletion within the first 20 years of life (healthy control group: Spearman´s r=0.1526, p= 0.4567; CD: Spearman´s r= 0.2539, p=0.1924). Both treated and untreated CD children (**Figure 6a**) as well as adult patients (**Figure 6b**) appeared to have normal circulating iNKT cell numbers as compared to healthy age-matched controls. Since large individual differences were observed, 4 individual patients with relatively low, intermediate and high iNKT levels were followed longitudinally before and 1 year after the start of the GFD. In all patients, the iNKT levels appeared to be remarkably stable (data not shown) during this year.

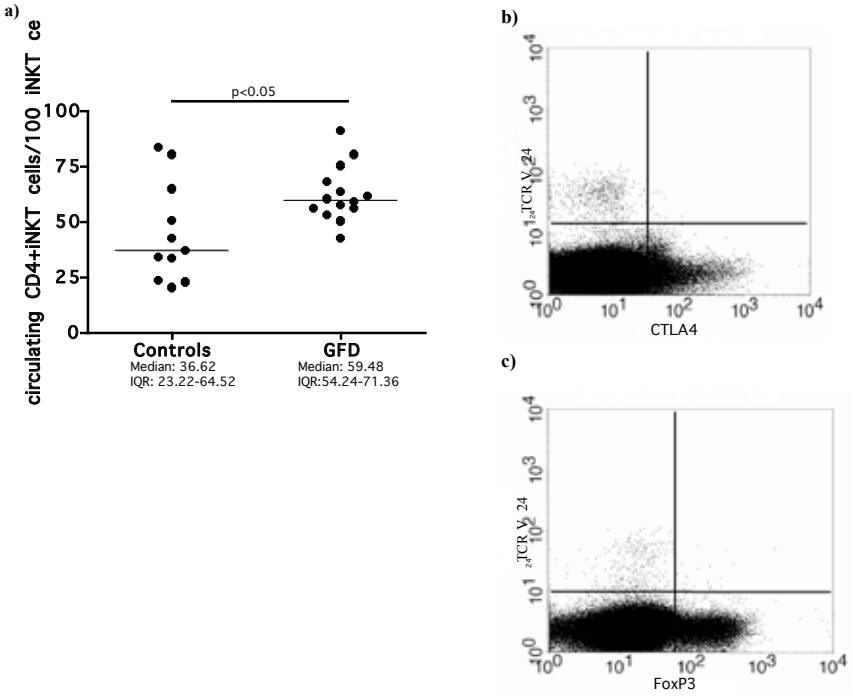

Figure 7 Analysis of iNKT cell phenotype. **a**) Percentage of circulating CD4+iNKT cells in peripheral blood of GFD responding CD patients (GFD) and healthy volunteers without known autoimmune diseases or malignancies (C). (two-tailed Mann-Whitney U test, p=0.0375). Horizontal bars indicate median values. IQR: interquartile range, n: sample size. **b**) staining of intracellular CTLA4 and **c**) FoxP3 in iNKT cells.

The regulatory phenotype of iNKT in CD

Although numbers of iNKT cells were unaffected in active and treated CD, we next investigated to what extent the iNKT subset (CD4+) with regulatory functions was affected in the different coeliac patients. Unfortunately, iNKT cell numbers of patients with RCD were too low to allow for accurate assessment of their phenotype. Therefore we compared the phenotype (CD4+ or CD4-) of the iNKT cells and their expression of the regulation associated molecules FoxP3 and CTLA4 in uncomplicated CD (on a GFD) and in age-matched healthy controls. CD patients on a GFD showed a higher proportion of CD4+ iNKT cells (**Figure 7a**, median 59%) than healthy controls (p=0.0375, median 37%). Regarding the expression of the Treg-associated proteins, **figure 7** illustrates that circulating iNKT cells in both CD patients and healthy controls, did not express CTLA4 or FoxP3, regardless of their CD4 expression (**Figure 7b and c**).

Discussion

iNKT cells are thought to be important in tumor immune surveillance as well as in the regulation of autoimmune disease [7, 23]. This view is not only supported by numerical defects in circulating iNKT cells in autoimmune disease and malignancy [17, 25], or because they protect against type 1 diabetes in NOD mice after interaction with Treg cells [32], but also by the fact that such reduced iNKT cell numbers were found to be associated with a poor prognosis in head and neck cancer [24]. It is, however, still a matter of debate whether a lack of adequate iNKT cells should be considered as a risk factor for, or rather as a consequence of the disease process. To address this question CD patients represent a unique population showing the whole pathogenetic spectrum from a state of gluten hypersensitivity with reversible villous atrophy to a state of gluten independent autoimmune disease in RCD and from RCD I with relatively good prognosis to RCD II with a high risk for malignant disease and eventually to the development of EATL [30]

Our results are compatible with the hypothesis that iNKT cells may prevent progression to RCD. However, to evaluate whether a numerical defect actually precedes a state of refractory disease, patients on a GFD with low iNKT cell numbers should be followed-up to assess their risk for developing RCD. Since only a very small proportion of patients on GFD develops refractory disease this will be a very inefficient approach. On the other hand, it is also clear from our results that other factors play a role, since low iNKT cell numbers are, at least in some patients, compatible with good responsiveness on a GFD.

Regarding the risk of progression from a state of refractory CD to the development of pre-malignant and malignant disease, we tested whether the iNKT defects would be related to the presence of aberrant IEL, a characteristic of RCD II patients, which is associated with a relatively short survival time [30]. No such relation was found, however, probably because of the already very low, often hardly detectable iNKT cell numbers in RCD patients. Moreover, in patients who developed EATL, no further depletion (if at all possible because of the already low levels) of circulating iNKT had occurred. Still,

our data suggest that the reduced numbers of iNKT cells in RCD somehow predispose for, or at least reflect a state of progressive, uncontrolled autoimmune destruction, since patients with active, untreated coeliac disease, both children and adults, had normal levels of iNKT cells as compared to age matched healthy controls. This is in line with earlier results from our group [17], but in contrast with reports from Grose et al [28], who described a peripheral numerical and functional deficiency of iNKT cells in both active and treated CD patients, accompanied by reduced iNKT cell numbers in the intestinal mucosa. It is not clear what causes this discrepancy, since both studies included relatively large numbers of patients, age matched controls and similar methodology. We evaluated the CD3+Vα24+Vβ11+ population in several clinical studies and found high correlations with αGalCer/CD1d tetramer-positive iNKT cells [33]. Furthermore, Grose et al [28] also found a significant reduction in Vα24+ Vβ11+ double positive cells, indicating that the discrepancy cannot be explained by different methodological approaches. The only difference remains in the fact that Grose et al [28] performed parametric statistical analyses although in the present study, iNKT cells did not have a normal distribution so non-parametric statistics were used in our study. We do not know whether concomitant Crohn´s Disease or Ulcerative Colitivis [27] or other autoimmune diseases, known to be associated with decreased iNKT numbers [17] and that had been excluded from our study have been included in that of Grose et al.
Finally, the phenotype of the iNKT cells was evaluated as support for a regulatory function of these cells. Unfortunately in RCD patients iNKT levels were too low to allow for accurate phenotyping. However, in CD patients responding to a GFD a higher frequency of CD4+ iNKT was found than in age matched healthy controls. Interestingly, gliadin-specific regulatory T cells were found in coeliac patients on a GFD as well [34], indicating that regulatory cell numbers can increase during a GFD, or that individuals with higher frequencies of regulatory cells are more likely to respond to a GFD. Remarkably we did not find molecular support for a regulatory function of the iNKT cells, since no expression of intracellular FOXP3 and CTLA4 was observed in either the CD4+ or CD4- iNKT subset. This might suggest a different regulatory mechanism in iNKT cells than in Tregs, although it cannot be excluded that these regulation- associated proteins can be induced in iNKT cells upon activation.
Treatment of RCD involves immunomodulating drugs [35, 36, 37] and, in RCD II, chemotherapy and autologous hematopoietic stem cell transplantation [38]. However, since none of these therapies has been fully effective so far, additional treatments supporting the homeostasis of the immune system could be of vital importance. Recently, Gianfrani et al [34] suggested a future therapeutic approach based on ***in vitro*** expanded gliadin-specific regulatory T cells with a specific gut-homing capacity, which could be reintroduced into RCD patients. Since we have clearly demonstrated a selective depletion of iNKT cells in RCD, new therapeutic strategies should also aim at reconstitution of adequate numbers of appropriately polarized iNKT cells [23].

With respect to other circulating regulatory T cells, like Treg and Tγδ cells, we did not find abnormal levels, neither in responsive nor in refractory CD. Also, no differences

were found between RCD I and II patients, although the RCD patient subgroups here were rather small. Tregs are, like iNKT cells, believed to prevent the development of autoimmune disease, while, on the other hand, tumors might benefit from them due to down regulation of anti-tumor immune responsiveness [39]. Despite normal levels in the circulation, however, defects locally in the intestinal mucosa with respect to numbers or regulatory capacity cannot be excluded. CD is known to be characterised by a permanent increase of TCRγδ IELs with a concomitant elevation of infiltrating TCRαβ+ cells during the active stage of the process [40, 41, 42]. However, the TCRαβ+ cells decrease in response to gluten withdrawal, whereas for TCRγδ+ cells this may take years to occur[43]. Relatively high levels of mucosal Tγδ [44, 45] and recently of gliadin specific regulatory T cells [34] have been described in CD in remission. In other intestinal diseases like colitis, CD4+CD25+ Tregs were shown to prevent disease development[46]. Therefore, future investigation of not only mucosal iNKT cells but mucosal Tregs and Tγδ cells as well might shed more light on their role in maintaining intestinal homeostasis in CD patients.

In summary, our study demonstrates that from the circulating regulatory T cells tested, only the iNKT cell numbers have become selectively reduced in coeliac disease refractory to the gluten free diet. New therapeutic strategies aiming at expansion of appropriately polarized iNKT cells [23] may be effective in preventing disease progression in refractory coeliac patients.

Acknowledgments

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9.
The MYO9B gene is a strong risk factor for the development of Refractory Coeliac Disease.

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Abstract

Background & Aims: Coeliac disease (CD) is associated with HLA-DQ2 and HLA-DQ8 and has been linked to genetic variants in the *MYO9B* gene on chromosome 19. HLA-DQ2 homozygosity is associated with complications of CD such as refractory coeliac disease type II (RCD II) and enteropathy-associated T-cell lymphoma (EATL). We investigated whether *MYO9B* also predisposes to RCD II and EATL.
Methods: Genotyping of *MYO9B* and molecular HLA-DQ2 typing were performed on 62 RCD II and EATL patients, 421 uncomplicated CD patients and 1624 controls.
Results: One single nucleotide polymorphism in *MYO9B* showed a significantly different allele distribution in RCD II and EATL patients compared to controls (p=.00002). The rs7259292 T allele was significantly more frequent in RCD II and EATL patients compared with CD patients (p=.0003, odds ratio [OR], 3.61; 95% confidence interval [CI] 1.78-7.31). The frequency of the haplotype carrying the T allele of this single nucleotide polymorphism was significantly increased in RCD II and EATL patients (11%), compared with controls (2%) and CD patients (3%) (OR, 6.76; 95% CI, 3.40–13.46; p=2.27E-09 and OR, 4.22; 95% CI, 1.95–9.11; p=.0001, respectively). Both *MYO9B* rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to CD patients (OR, 4.3; 95% CI, 1.9–9.8 and OR, 5.4; 95% CI, 3.0–9.6, respectively), but there was no evidence for interaction between these two risk factors.
Conclusions: We show that both *MYO9B* and HLA-DQ2 homozygosity might be involved in the prognosis of CD and the chance of developing RCD II and EATL.

Introduction

Coeliac disease (CD) is an immune-mediated enteropathy following the ingestion of gluten. It is characterized by a permanent intolerance for gluten proteins present in dietary wheat, rye and barley. The pathogenesis of disease involves interactions between environmental, genetic and immunologic factors.[1;2]
Genes play a key role in the pathogenesis of CD. The class II HLA-DQ2 and HLA-DQ8 loci are the most important genetic contributors identified so far. The HLA DQ2 heterodimer is encoded by the DQA1*0501 and DQB1*02 alleles and is present in 95% of all individuals diagnosed correctly with CD. Almost all the remaining patients express HLA-DQ8.[3-8] These HLA molecules are necessary to develop the disease but are not sufficient for the phenotypic expression. HLA explains only 40% of the heritable risk, so non-HLA genes must also be involved in CD.[9-11] Several studies found evidence for linkage to regions on different chromosomes, including chromosomes 5, 6 and 19.[12-16]
Our group found strong evidence for linkage to genetic variants on chromosome 19, in the myosin IXB gene (*MYO9B*), which might lead to an impaired intestinal barrier and thereby play a role in the pathogenesis of CD.[17] Individuals homozygous for the at-risk *MYO9B* allele had a 2.3 times higher risk of CD.
So far, the only treatment for CD is a life-long gluten-free diet (GFD). However, a small subgroup (2-5%) of CD patients diagnosed at adult age fails to improve on a

GFD.[18] In this group of patients, the enteropathy persists despite adherence to the diet or recurs after an initially good response to the diet. These patients are regarded as suffering from refractory coeliac disease (RCD), defined as persisting villous atrophy with crypt hyperplasia and increased IELs despite maintaining a strict GFD for more than 12 months, or when severe symptoms necessitate intervention independent of the duration of the GFD.[19-23] With immunephenotypical analysis, two types of RCD can be recognized; RCD I and RCD II. RCD II patients are characterized by the presence of aberrant (CD7+CD3-CD4-CD8- cytoplasmic CD3+) IELs in the small bowel mucosa, whereas these lymphocytes are not detected in RCD I patients.[23-25] Approximately half of the RCD II patients develop an enteropathy-associated T-cell lymphoma (EATL) within 5 years, whereas RCD I patients seldom develop an EATL.[26;27]
In the RCD II and EATL group, significantly more patients were seen to be HLA-DQ2 homozygous compared with uncomplicated CD patients. This difference was even more pronounced when compared with healthy controls.[28]
Our aim of this study was to investigate whether the *MYO9B* gene is an additional risk factor for the development of RCD II and EATL, similar to HLA-DQ2 homozygosity. We also investigated a possible interaction between *MYO9B* and HLA.

Patients and methods

subjects

RCD II patients: A total of 62 Dutch white RCD II and EATL patients were included in the study (50 RCD II patients and 12 EATL patients). All patients were ill and had severe malabsorption; RCD II is difficult to treat. More than half of the RCD II patients have since died. Patients were treated with Cladribine therapy (ref. Al Toma CGH 2006) and 8 patients underwent autologous stem cell transplantation with good result (ref.Al Toma Blood 2007). However, 25 (52%) of the RCD II patients went on to develop EATL. The 2-year survival rate for EATL patients is 15-20%, despite aggressive treatment with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and alemtuzumab, and 4 patients even had autologous stem cell transplantation, but with disappointing results in contrast to the 8 RCD II patients. All except one of our patients were also included in a former study by Al Toma et al[29]. Because our main interest was to look for genetic association in RCD II patients, we refer to our cohort of RCD II and EATL patients as RCD II. The diagnosis of CD was confirmed by histological examination, with a documented histological response to gluten withdrawal.[23;30] All patients and their adherence to GFD were regularly checked in the outpatient clinic by a dietician. Patients with CD were considered to be refractory if their symptoms of malabsorption caused by villous atrophy persisted despite strict adherence to a GFD or recurred after an initially good response to the diet. Their histopathology showed at least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria and after excluding other possible causes of villous atrophy.[23;31]
Additional information about the diagnosis, evaluation, HLA typing, and IEL phenotyping is given in **table 1** and in the supplementary information on RCD II patients.
Patients with Coeliac Disease: A total of 421 Dutch white subjects with uncomplicated

Patients Characteristic	Total group	RCD-II Total	EATL after RCD-II	EATL only
Total (Male: Female)	62 (29:33)	50 (19:31)	25 (10:15)	12 (10:2)
Age in years at diagnosis CD (±SD) (range)	56 (± 10.2) (33-74)	54 (±10.6) (33-74)	55 (± 9.4) (34-71)	63 (± 3.8) (56-70)
Age in years at diagnosis RCD II / EATL (±SD) (range)	58 (±8.5) RCDII 62 (±7.4) EATL (39-79)	58 (±8.5) (39-74)	61 (±8.6) (52-79)	64 (±4.0) (56-70)
DQ2 Total -Heterozygosity -Homozygosity	60 (96%) 22 (35%) 38 (61%)	49 (98%) 19 (38%) 30 (60%)	24 (96%) 5 (20%) 19 (76%)	11 (92%) 3 (25%) 8 (67%)
Myosin IXB -Heterozygosity	12 (19%)	10 (20%)	4 (16%)	2 (16%)

Table 1. Baseline demographic characteristics of refractory coeliac disease (RCD II) groups

CD were included in the study. They were a subgroup of CD cases described previously[32], but from which we excluded all individuals with RCD I (n=22), RCD II (n=9), RCD II and EATL (n=10), RCD II and ulcerative jejunitis (n=1) and EATL (n=1). HLA data were available for 407 individuals.

Controls: A total of 1624 Dutch white controls were included in the study. The control group comprised the controls used in the article of Monsuur *et al*[33] expanded with 938 extra blood bank controls.[34] The total control cohort (n=1624) comprised blood donors from Amsterdam (n=429), Leiden (n=475) and Utrecht (n=500) and healthy spouses from different, non-autoimmune projects (n=220). HLA data was available for 477 controls.

HLA typing

Whole blood was obtained from CD and RCD II patients for typing of HLA-DQA1* and DQB1* alleles, performed with a combined single-stranded conformation polymorphism (SSCP)/heteroduplex (HD) method by a semi-automated electrophoresis and gel staining method on the Phastsystem™ (Amersham Pharmacia Biotech, Sweden). Individuals were designated HLA-DQ2 if alleles DQA1*0501 and DQB1*02 were present, and HLA-DQ8 if alleles DQA1*03 and DQB1*0302 were present.[35-37] We did not examine the compound heterozygote of DQA1*05 and DQA1*0201 with DQB1*0201 and DQB1*0202 and we have not classified this combination.

In controls, DQA1, DQB1 and DRB1 typing was performed by polymerase chain reaction with sequence-specific biotin-labeled oligonucleotides as described.[38] HLA data of a part of the control patients was collected from the ITItwo panel (ITI panel is a DNA panel from the Section Immunogenetics and transplantation Immunology and consist of 477 unrelated randomly selected, Dutch blood donors).

		Healthy controls		RCD II patients				
SNP	Maj/min allele	Maj.allele (freq)	Min.allele (freq)	Maj.allele (freq)	Min.allele (freq)	P=value	OR	95% CI
rs7259292	C_T	3119 (0.97)	91 (0.03)	112 (0.90)	12 (0.10)	0.00002	3.90	2.10 - 7.25
rs2305767	A_G	1764 (0.56)	1392 (0.44)	79 (0.65)	43 (0.35)	0.05	1.43	0.99 - 2.09
rs1545620	A_C	2056 (0.64)	1148 (0.36)	67 (0.55)	55 (0.45)	0.04	1.47	1.03 - 2.12
rs1457092	C_A	2120 (0.67)	1042 (0.33)	77 (0.63)	45 (0.37)	0.37	1.20	0.83 - 1.74
rs8107108	C_T	2959 (0.92)	245 (0.08)	110 (0.90)	12 (0.10)	0.37	1.41	0.77 - 2.57
rs2305766	G_C	2114 (0.67)	1050 (0.33)	76 (0.62)	46 (0.38)	0.30	1.23	0.85 - 1.78
rs2305764	G_A	1958 (0.62)	1204 (0.38)	71 (0.60)	47 (0.40)	0.70	1.08	0.75 - 1.58
rs2279002	A_G	2263 (0.71)	933 (0.29)	81 (0.66)	41 (0.34)	0.30	1.24	0.85 - 1.82

Table 2a. Allele distribution of *MYO9b* SNPs in RCD II patients (n=62) compared to healthy controls (n=1624)

		CD patients		RCD II patients				
SNP	Maj/min allele	Maj.allele (freq)	Min.allele (freq)	Maj.allele (freq)	Min.allele (freq)	P=value	OR	95% CI
rs7259292	C_T	815 (0.97)	25 (0.03)	112 (0.90)	12 (0.10)	0.0003	3.61	1.78 - 7.31
rs2305767	A_G	508 (0.62)	316 (0.38)	79 (0.65)	43 (0.35)	0.51	0.88	0.59 - 1.31
rs1545620	A_C	485 (0.58)	349 (0.42)	67 (0.55)	55 (0.45)	0.50	1.14	0.78 - 1.67
rs1457092	C_A	510 (0.61)	328 (0.39)	77 (0.63)	45 (0.37)	0.63	0.92	0.62 - 1.35
rs8107108	C_T	774 (0.93)	58 (0.07)	110 (0.90)	12 (0.10)	0.26	1.54	0.81 - 2.92
rs2305766	G_C	508 (0.62)	314 (0.38)	76 (0.62)	46 (0.38)	0.92	0.99	0.67 - 1.46
rs2305764	G_A	445 (0.54)	381 (0.46)	71 (0.60)	47 (0.40)	0.20	0.78	0.53 - 1.15
rs2279002	A_G	546 (0.66)	282 (0.34)	81 (0.66)	41 (0.34)	0.92	0.99	0.66 - 1.48

Table 2 b. Allele distribution of *MYO9b* SNPs in RCD II patients (n=62) compared to uncomplicated CD patients (n=421)

Single Nucleotide Polymorphism Selection

Eight tag single nucleotide polymorphism (SNPs) (see **table 2**) completely tagging the 3' region of *MYO9B* (from rs7259292-rs388484) were typed.[39] Three of these SNPs (rs2305767, rs2305764 and rs1457092) were also used by Monsuur *et al.*[40;41]

SNPs were genotyped using Taqman assays (Applied Biosystems, Foster City, California, USA) and were performed according to the manufacturer's specifications. Genotypes were analyzed using a TaqMan 7900HT (Applied Biosystems). In the controls, the frequency of all SNPs was in Hardy-Weinberg equilibrium (p>0.05).

Statistical analysis

The haplotype structure and linkage disequilibrium in the region were investigated with HAPMAP data (a haplotype map of the human genome, www.hapmap.org[42] with the Haploview application.[43] Allele and genotype distributions in cases and controls were compared with the COCAPHASE module of the UNPHASED statistical package.[44] Haplotype association was estimated using the same package. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in 2 x 3 χ^2 table. Odds ratios (OR) were calculated, and the confidence intervals were approximated by using Woolf's method with Haldane's correction.[45]

To investigate the added value of *MYO9B* to HLA-DQ2 homozygosity, we used a logistic regression model. First, HLA-DQ2 homozygosity (no/yes) was added to the model in which we compared RCD II patients to CD patients. Then we added the *MYO9B* variant (combining the heterozygous and the homozygous variants) that was most strongly associated to the disease and tested whether the model improved by using a likelihood ratio test. We also evaluated whether the *MYO9B* variant was independently associated to the risk of RCD II compared with CD patients by evaluating the OR and 95% CI. We tested for possible interaction between HLA-DQ2 homozygosity and the *MYO9B* variant by including the interaction term in the logistic regression model. We used Stata (Stata/SE 8.2 for Windows, StataCorp LP, College Station, TX, USA) for these analysis.

Results

We observed that SNPs rs7259292 showed a different allele distribution in RCD II patients compared with the control group,.with minor allele frequencies of 10% in RCD II patients compared with 3% in controls (p=0.00002, OR 3.90, 95% CI: 2.10 – 7.25) (**Table 2a**). The association of rs1545620 with RCD II compared with controls was borderline statistically significant (OR, 1.47; 95% CI, 1.03-2.12; P=.04) (**Table 2**).

All tested SNPs were located in 1 haploblock and were in strong linkage disequilibrium with each other. We therefore continued by constructing 8-SNP haplotypes. Five haplotypes occurred with a frequency of more than 5% in RCD II cases or controls. Haplotype h5, which carries the associated rs7259292*T allele, occurred significantly more frequently in RCD II patients than controls (11% vs 2%, p=2.27E-09, OR 6.76, 95% CI 3.40 – 13.46) (**Table 3**).

On comparing the allele frequencies of the 8 tested SNPs in Myo9B between RCD II patients and CD patients, we observed a statistically significant difference in the frequency of rs7259292, as well as in the frequency of haplotype h5 in RCD II patients compared with CD patients (p=0.0003, OR 3.61, 95% CI 1.78 – 7.31 and p=0.0001, OR 4.22, 95% CI 1.95 – 9.11 on a single SNP and haplotype h5, respectively) (**Table 2b, Table 3**).

		RCD II patients	Controls	RCD II patients compared to controls			CD patients	RCD II patients compared to CD		
	Haplotype*	count (freq)	Count (freq)	p-value	OR	95% CI	count (freq)	p-single haplotype	OR	95% CI
h1	C-A-A-C-C-G-G-A	10 (0.09)	223.9 (0.08)	0.26	1.58	0.79 - 3.18	52.56 (0.07)	0.33	1.53	0.73 - 3.22
h2	C-A-A-C-T-G-G-A	9.33 (0.08)	228.2 (0.08)	0.41	1.46	0.71 - 2.98	50.37 (0.07)	0.38	1.50	0.70 - 3.21
h3	C-A-C-A-C-C-A-G	32.91 (0.29)	824.7 (0.29)	0.27	1.33	0.83 - 2.13	251.4 (0.33)	0.99	1.01	0.61 - 1.64
h4	C-G-A-C-C-G-G-A	37.65 (0.34)	1249 (0.43)	ref	1	-	287.3 (0.37)	ref	1	-
h5	T-A-C-C-C-G-G-A	12 (0.11)	61.2 (0.02)	2.27E-09	6.76	3.40 - 13.46	21.99 (0.03)	0.0001	4.22	1.95 - 9.11
	overall p-value			0.0004				0.002		

Table 3 Haplotype analysis of myosin IXB SNPs in RCD II patients (n=62), compared to health controls (n=1624) and uncomplicated CD patients (n=421)
* the order of SNPs is as follows: rs7259292, rs2305767, rs1545620, rs1457092, rs8107108, rs2305766, rs2305764, rs2279002

Groups studied	CC (freq)	CT (freq)	TT (freq)	Allele C(freq)	Allele T(freq)
Controls (n=1624)	1517 (0.95)	85 (0.05)	3 (0.00)	3119 (0.97)	91 (0.03)
Controls HLA-DQ2 homoz (n=10)	10 (1.00)	0 (0.00)	0 (0.00)	20 (1.00)	0 (0.00)
CD (n=421)	396 (0.94)	23 (0.05)	1 (0.00)	815 (0.97)	25 (0.03)
CD HLA-DQ2 homozygous (n=97)	93 (0.96)	4 (0.04)	0 (0.00)	190 (0.98)	4 (0.02)
RCD II (n=62)	50 (0.81)	12 (0.19)	0 (0.00)	112 (0.90)	12 (0.10)
RCD II HLA-DQ2 homozygous (n=38)	30 (0.79)	8 (0.21)	0 (0.00)	68 (0.89)	8 (0.11)
RCD II non-HLA-DQ2 homozygous (n=23)	19 (0.83)	4 (0.17)	0 (0.00)	42 (0.91)	4 (0.09)

Table 4 a. Distribution of genotypes and alleles of rs7259292 SNP in groups studied b. Logistic regression model for HLA-DQ2 homozygosity and the presence of rs7259292*T allele in RCD II patients compared to uncomplicated CD patients

Risk factors	P>|z|	OR	95% CI
HLA-DQ2 homozygous	<0.000	5.4	3.0 – 9.6
rs7259292*T allele	<0.000	4.3	1.9 – 9.8

Table 4b. Logistic regression model for HLA-DQ2 homozygosity and the presence of rs7259292*T allele in RCD II patients compared to uncomplicated CD patients

Strong association of RCD II and EATL with homozygosity for the HLA DQ2 allele (DQA1*0501 and DQB1*0201) was recently reported by Al-Toma et al.[46] In the cohort of RCD II patients included in our study, 38 out of 61 HLA-typed patients (62%) were DQ2 homozygous (DQA1*0501 and DQB1*0201), whereas only 97 out of the 407 HLA-typed CD patients (24%) and 10 out of the 477 HLA typed controls (2%) carried 2 copies of the DQ2 allele. The OR for RCD II patients compared with uncomplicated CD was 5.2 (95% CI, 2.9 – 9.0; p=.27E-10). Compared to controls the OR was 69.1 (95% CI, 31.1 – 153.6; p=2.16E-54). The frequencies of individuals who carry both HLA-DQ2 homozygosity and rs7259292*T are higher in our patient cohort (**Figure 1**). Both *MYO9B* rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to CD patients (OR, 4.3; 95% CI, 1.9–9.8 and OR, 5.4; 95% CI, 3.0–9.6, respectively) and we found no evidence for interaction between these 2 risk factors.

We performed a logistic regression model including both HLA-DQ2 homozygosity and the presence of rs7259292*T allele, comparing RCD II patients with CD cases, and observed that both HLA-DQ2 homozygosity and the presence of rs7259292*T allele are independently associated with the development of RCD II (**Table 4b**). In the likelihood ratio test, the model fit improved when the rs7259292*T allele was included in the model subsequent to HLA-DQ2 homozygosity (p<0.0009).
To test for possible interaction between HLA-DQ2 homozygosity and rs7259292 genotype, we included an interaction term in the model. It was not statistically significant, and we therefore concluded that there was no statistical interaction.

Discussion

We found a strong association of myosin IXB rs7259292 SNP with the RCD II and EATL patients, referred to as RCD II here. The frequency of the minor allele was increased to 10% in RCD II patients, compared to 3% in controls and 3% in uncomplicated CD patients. Although the risk associated with the minor allele is high, the impact of these alleles at the population level is relatively small because of the low allele frequency (population attributable risk, 7%). The frequency of the associated but rare rs7259292*T allele was also higher compared with uncomplicated CD patients, and this was also observed at the haplotype level. We also found that the presence of the susceptible rs7259292*T allele did not interact with the HLA risk.

RCD is usually considered as a complication of CD, affecting only a small proportion (2-5%) of CD patients, almost all of whom were diagnosed at older than the age of 50 years.[47] RCD II is characterized by the development of aberrant T-cells, high frequency of EATL, and poor prognosis.[22;48-50] Finding genetic markers that could predict predisposition to RCD II among the CD population is of great interest: the subgroup of CD patients identified with a high risk for RCD II could undergo regular endoscopy with the aim of detecting T-cell lymphoma at an early stage and providing early treatment.

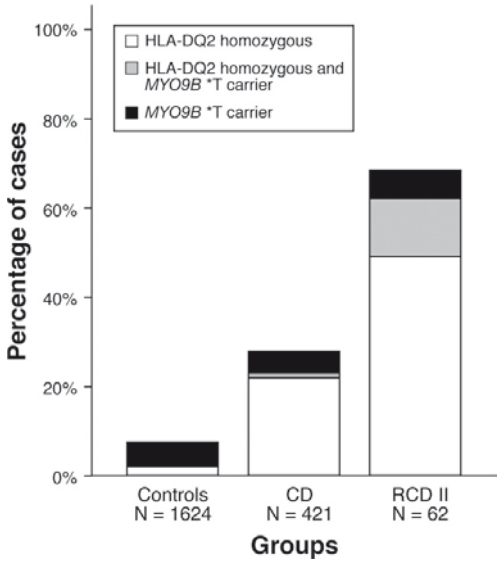

Figure 1. Distribution of HLA-DQ2 and MYO9B risk alleles in controls, coeliac disease patients and RCD type II individuals.

The most important risk factor for the development of RCD II known to date is the presence of two copies of the HLA-DQ2 haplotype.[26;27] By comparing RCD II patients with a group of uncomplicated CD patients without RCD, we have discovered that the presence of the susceptible *MYO9B* rs7259292*T allele is another genetic risk factor for the development of RCD II, independent and comparable to HLA. Nearly 70% of RCD II patients carry 2 copies of the HLA-DQ2, the *MYO9B* rs7259292*T allele, or both, compared to 7.5% in controls and 27.7% in uncomplicated CD patients (**Figure 1**). We do not have access to RCD groups from other populations and cannot determine whether this observation is specific to our Dutch cohort.

We can only speculate why MYO9B might underlie the mechanism of RCD. The RCD-associated SNP lies in a different part of the gene (intron 14) when compared with the variant associated to uncomplicated CD (intron 28). The possible differences of association might point to differences in disease mechanisms. We speculate that the RCD-associated variant changes the cytoskeleton, for example, specifically int T-cells that might result in abnormal T-cell responses and ultimately in clonal expansion of T-cells. Much functional work is required to determine the answer.

Rs7259292 is a rare SNP, with a frequency of 3% in the control population and in uncomplicated CD patients. Because the frequency of this SNP is 4,2% in the hapmap-CEU population, we do not think that the Dutch population is at especially high risk of RCD II. The SNP rs7259292 is located deep in the intron, not in a conserved sequence. According to the splicing prediction program (http://www.tigr.

org/tdb/GeneSplicer/gene_spl.html), it does not create any alternative splicing sites. It is therefore unlikely that the SNP itself is the causal mutation, but rather that it is a marker of disease in linkage disequilibrium with a causative variant. We therefore investigated which SNPs in the myosin IXB region were in strong linkage disequilibrium (have high correlation, i.e. r^2) with the rs7259292. We found two SNPs with an r^2 greater than 0.8; rs16981660 and rs11879844. Both are located in the introns and also do not potentially influence splicing. However, since the HAPMAP data is far from complete, we believe that other SNPs located on the same haplotype might be the causative variants. Fine-mapping of this region in the course of our study was not possible because of the strong linkage disequilibrium in the region and a low number of patients. Rs7259292 could also be in linkage disequilibrium with polymorphisms other than SNPs, such as deletions, insertions or repeats. Sequence analysis of the *MYO9B* gene in RCD II patients who carry the susceptible h5 haplotype could lead to identifying the functional variant.

Previously, the rs2305764 SNP was shown to be strongly associated with uncomplicated CD in the Dutch population.[51] Excluding the 42 RCD patients from the original study by Monsuur et al did not change these results (data not shown). But this could not be confirmed in CD populations in the United Kingdom, Spain, Italy and Scandinavia.[52-54] This discrepancy raises some doubts on the influence of this gene on predisposition to CD. The limited replication of *MYO9B* association with CD in other populations reduced the validity of this gene and makes it difficult to interpret the true role of *MYO9B* in CD pathogenesis, although, a positive association was recently found between *MYO9B* gene polymorphisms and CD and two other auto-immune diseases in a Spanish population.[55]
Furthermore, the replication studies mentioned included only the 3 most associated SNPs from the original study in Dutch population, whereas our results show an association of RCD II with a rare MYO9B haplotype, different from the CD associated haplotype. This might imply genetic heterogeneity between CD and RCD II in the Dutch population and might suggest that different Myo9B variants could be associated with CD in other populations.
In other auto-immune mediated intestinal diseases, such as Crohn's disease, genetic association studies have also reported conflicting results. Allelic variants in NOD2/CARD15 and DLG5 have been found to be associated with Crohn's disease susceptibility.[56-59] Both association differed among various populations and could not be confirmed in all populations.[60-67]

We can speculate that certain loci might be specific to a population and only relate to a specific phenotype of the disease, for example, as in the development of complicated CD. It is tempting to speculate that rs2305764 is associated with the development of RCD II or EATL. For example, in inflammatory bowel disease, the *IBD5* locus has been identified as a determinant of disease susceptibility as well as disease severity.[68]

We realize that our study has limitations, the most important of which is the relatively small number of patients (n=62). However, RCD II and EATL are rare complications seen in only 2%-5% of CD patients, and there are, as yet, no large cohorts of these complicated CD patients. The control group is proportionally extremely high (n=1624). The MYO9B genotypes for 938 extra controls were already available from the previous studies.[69] Frequencies of all MYO9B SNPs were similar in both the initial control group[70] and in 938 extra controls. We investigated the frequency of rs7259292 SNP in subgroups of our control cohorts but observed similar frequencies in all the groups. The differences in frequency of rs7259292*T was significant when RCDII group was compared with any particular subgroup of controls (**Table 1** in supplementary information).
Transmission disequilibrium test analysis would be relevant to confirm the results of the case-control analysis, however typing parents of RCD patients is almost impossible, given that the mean age of these patients is 56 years. Besides, the patients were diagnosed several years ago (since 1992).

Our study showed that not only HLA-DQ2 homozygosity but also SNP rs7259292 in the *MYO9B* gene are associated with an increased risk of developing RCD II and EATL. It would be interesting to design a prospective study to investigate the positive predictive value of both HLA-DQ2 homozygosity and *MYO9B* rs7259292*T carriership for RCD II and EATL prognosis and subsequent disease management.

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Supplementary information on RCD II patients

Diagnostic criteria

The diagnosis of CD was confirmed by histological examination with a documented histological response to gluten withdrawal.[23;71] Patients with CD were considered to be refractory (RCD) when (1) their symptoms of malabsorption due to villous atrophy persisted despite strict adherence to a gluten-free diet (GFD) or recurred after an initially good response to the diet; and (2) the histopathology showed at least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria and after other possible causes of villous atrophy had been excluded.[23;72;73]

Two types of RCD can be distinguished immunologically in the small bowel mucosal, depending on the presence or absence of T-lymphocytes with an abnormal phenotype. In RCD I, the intra-epithelial lymfocyte phenotype is normal with the expression of surface CD3, CD8 and T-cell receptors (TCR). In RCD II, the IELs have normal morphological features, but they exhibit an aberrant IEL phenotype despite the normal expression of CD103 and CD7. They also show downregulation of surface CD3 to intracytoplasmic CD3, and lack of classical surface T-cell markers such as CD4, CD8 and, as a consequence of the CD3 downregulation, lack of surface TCR expression.[74;75] Using immunophenotyping flow-cytometric analysis of the intestinal mucosal, we arbitrarily chose to regard ≤10% of aberrant cells as normal, and >20% as abnormal, for lack of solid data in the literature. We included only patients with RCD II in our study as they are a well-defined group.

Evaluation

Clinical, laboratory (hematology, biochemistry and serology), endoscopic and histological examination of the small intestine was performed at regular 6-month intervals. Patients were checked at the outpatient clinic at regular intervals (3-6 months) and their adherence to the GFD was monitored. Particular attention was paid to symptoms and signs of malabsorption, body mass index and performance status. Antiendomysium antibodies and anti-tissue transglutaminase antibodies were tested at diagnosis and at follow-up in all patients. Immunophenotyping of IELs was performed on all patients. Endoscopy using upper gastrointestinal endoscopy, VCE and/ or DBE with small intestinal biopsies, CT scan, magnetic resonance enteroclysis, PET scan and dual energy X-absorptiometry were performed to check for and exclude EATL development.[23]

Small intestinal biopsies

Upper gastrointestinal endoscopy was performed in all RCD II patients. At least 10 duodenal biopsies were taken for histological, immunohistochemical and flow cytometric examination. Four to six biopsies were fixed and preserved in 10% formalin for histopathological and immunohistochemical evaluation. Three or four biopsies for TCR gene rearrangement studies were taken separately, preserved on histocon and frozen at –20°C. Another three or four biopsies were taken for immunophenotypical evaluation and preserved in RPMI medium.

Isolation of IEL and cell-staining for immunophenotyping

Lymphocytes and enterocytes were isolated from three or four small intestinal biopsies (SIBs) by homogenizing tissue samples and passing fragments through a 100µm nylon cell strainer (Becton Dickinson, Cell strainer) in RPMI medium supplemented with 1% FCS. The released cells were subsequently washed and labeled by 4-color staining for 30 minutes on ice with various combinations of fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein and allophycocyanin labeled monoclonal antibodies against CD3, CD4, CD8, CD7, CD103, CD19, CD45, CD16/56, γδTCR and cytoplasmic CD3. The FACS method used in this study was in accordance with the manufacturer's guidelines.

Cell surface immunophenotyping of IEL was performed on a 4-color FACS Calibur flow cytometer (Becton Dickinson, BD, immunocytometry systems, San Jose, CA). Non-viable cells and debris were excluded based on forward and sideways light scatter properties and a gate on CD45 positive cells was used for selecting lymphocytes. Intraepithelial localization of lymphocytes was confirmed by surface expression of CD103 (αEβ7 integrin, a gut homing receptor for E-cadherin). IELs were analyzed, using CellQuesttm (KS Stat) based on their expression of cell markers: cytoplasmic CD3, surface CD3, CD4, CD7, CD8, CD16/56, CD19, CD103 and TCRγδ on CD45+gated IELs. A level of aberrant cells (Cytoplasmic CD3+ surface CD3- % of lymphocytes) of ≤10% was regarded as normal, and >20% as abnormal.

Assessment of TCR gene rearrangement by Polymerase Chain Reaction (PCR)

DNA was extracted from cryosections of duodenal biopsies by a standard procedure using proteinase-K digestion and ethanol precipitation of the genomic DNA. T-cell receptor (TCR)- gamma (TCR-γ) gene rearrangements were analyzed by multiplex PCR amplification under standardized conditions. A monoclonal and polyclonal control were included in each experiment. Clonality assessment for TCR-γ gene rearrangements was done using the BIOMED-2 multiplex TCR PCR protocol.[76;77]

group	total ind	frq rs7259292*T	p-value	OR	CI
RCDII	62	0.097	n/a	1	-
LabCo	220	0.026	5.00E-04	3.99	1.74 - 9.12
AmsCo	429	0.028	1.00E-04	3.82	1.88 - 7.77
LeiCo	475	0.027	6.90E-05	4.02	1.99 - 8.13
UtrCo	500	0.03	2.00E-04	3.58	1.80 - 7.12

Table 1 Association of RCDII with rs7259292*T allele when compared separately to 4 subgroups of controls used in the study.

Part four
Treatment

10. Alemtuzumab for Refractory Coeliac Disease Type II might be disappointing

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Vivas et al (June 8th issue)[1] reported a patient with refractory coeliac disease (RCD) type II responding to alemtuzumab. However, from their report it is not clear whether the percentage of aberrant intraepithelial lymphocytes (IELs) actually decreased after therapy with alemtuzumab, as only the percentage of oligoclonal γδ+ T-cells is mentioned. This is of importance, as the RCD II-specific aberrant T-cell population, expressing CD7 and cytoplasmic CD3 while lacking the surface CD3/TCR complex and CD4/8, determines the risk for enteropathy-associated T-cell lymphoma (EATL). This population is distinct from the γδ T-cell population.[2] Herein, we report a patient showing an increase in aberrant IELs despite monoclonal anti-CD52 (alemtuzumab) treatment, whereas the small population of γδ T-cells was not affected.

A 66-year old woman had a resistant RCD, despite a gluten free diet and therapy with a combination of prednisone and cladribine. Duodenal biopsy showed persistent villous atrophy with crypt hyperplasia and increased IELs. Flow cytometry of IELs revealed 60% expressing an aberrant phenotype. Only 1% of γδ T-cells could be detected.

It was decided to start treatment with alemtuzumab. Although the patient showed a clinical remission as determined by an increase in weight and decrease in diarrhea, the mucosal lesions persisted and the aberrant IELs increased to 91%. Moreover, she developed skin lesions showing aberrant T-cells. Accordingly, clinical remission with persistent aberrant IELs has been described by Lundin et al. [3] A possible explanation might be that IELs are not effectively targeted by alemtuzumab, given the fact that in our patient almost all aberrant T-cells in the intestinal mucosa still expressed CD52 (figure 1), whereas in peripheral blood barely any (CD52 positive) lymphocytes could be detected.

Considering the fact that the majority of RCD II patients develops EATL, it seems justified to aggressively treat patients with aberrant IELs.[4] Although treatment with alemtuzumab appeared effective in the patient described by Vivas et al., aberrant T lymphocytes in the intestinal mucosa might not have been affected. Therefore, in future studies IEL phenotype should be investigated in addition to clinical and histological remission. Analysis of γδ-T-cell oligoclonality may be less relevant, as oligoclonal T-cells can also be detected in patients that do not develop EATL.[4;5] Thus, in order to determine whether alemtuzumab reduces the risk for EATL in RCD II patients, flow cytometric analysis to monitor aberrant IELs should be performed.

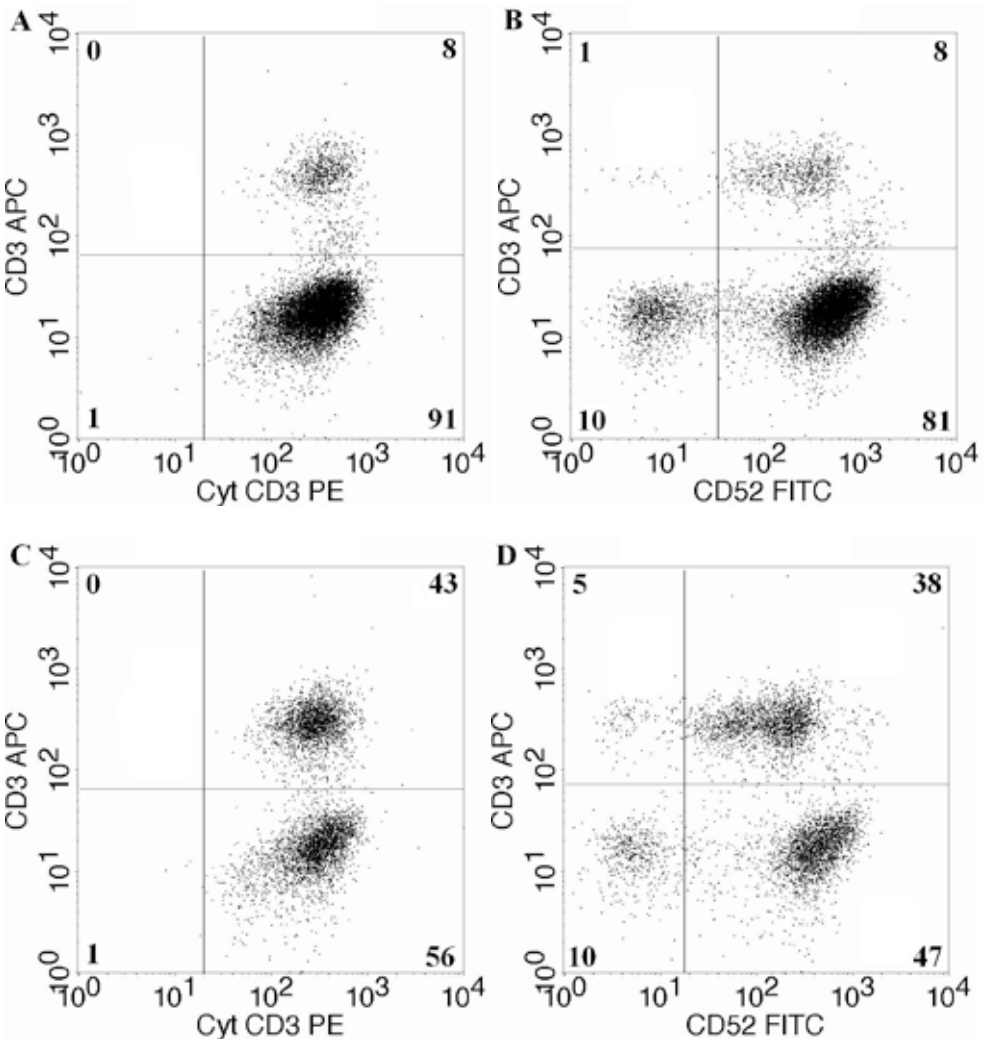

Figure 1: Flow cytometric analysis of lymphocyte populations isolated from duodenal biopsies after alemtuzumab treatment. **Panel A** depicts IELs showing that 91% displays an aberrant phenotype (surface CD3- cytoplasmic CD3+). **Panel B** shows that the the majority of aberrant IELs still expresses CD52 (81% of total IELs). **Panel C** depicts lamina propria derived lymphocytes (LPLs) of which 56% displays and aberrant phenotype. **Panel D** shows that the majority of aberrant LPLs still expresses CD52 (47% of total LPLs). All analyses were performed within the CD45+ CD7+ lymphocyte gate, using IEL and LPL populations that were isolated by standard biopsy dissociation procedures.

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11. Autologous hematopoietic stem cell transplantation in Refractory Coeliac Disease with aberrant T-cells.

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Abstract

Autologous hematopoietic stem cell transplantation (ASCT) is an increasingly accepted treatment for refractory autoimmune diseases. Refractory coeliac disease with aberrant T cells (RCD-type II) is unresponsive to available therapies and carries a high risk of transition into enteropathy associated T-cell Llymphoma (EATL). This study reports on the feasibility, safety and efficacy of ASCT in RCD type II.

Thirteen patients with RCD type II were evaluated. Seven patients [4 men, 3 women, mean age 61.5 years (range, 51-69 years)] underwent transplantation. After conditioning with fludarabine and melphalan, ASCT was performed. Patients were monitored for response, adverse effects and hematopoietic reconstitution.

All 7 patients completed the mobilization and leucopheresis procedures successfully and subsequently underwent conditioning and transplantation. Engraftment occurred in all patients. No major non-hematological toxicity or transplantation-related mortality was observed. There was a significant reduction in the aberrant T cells in duodenal biopsies associated with improvement in clinical wellbeing, and normalization of hematological and biochemical markers (mean follow-up, 15.5 months; range 7-30 months). One patient died 8 months after-transplantation form progressive neurocoeliac disease.

These preliminary results showed that high-dose chemotherapy followed by ASCT seems feasible and safe and might result in long-term improvement of RCD II patients whose condition did not respond promptly to available drugs. (Blood. 2007; 109:2243-2249)

Introduction

Autologous hematopoietic stem cell transplantation (ASCT) is an increasingly accepted effective treatment option for patients with severe autoimmune diseases refractory to conventional treatment [1] and has been used successfully in patients with multiple sclerosis,[2] rheumatoid arthritis,[3] systemic sclerosis [4], systemic lupus erythematosus [5] and Crohn's disease.[6] The rationale for this strategy is based on the concept of immunoablation by intense immunosuppression using-high dose chemotherapy, with subsequent regeneration of naïve T lymphocytes derived from reinfused hematopoietic progenitor cells.[7]

In coeliac disease (CD), HLA-DQ molecules bind and present gluten peptides to antigen-specific T-cells. These HLA-DQ-peptide complexes induce inflammatory responses in the small intestine consisting of lymphocytic infiltration of the lamina propria, expansion of the intraepithelial lymphocyte population, hyperplasia of the crypts and atrophy of the villi.[8] In a small percentage (2%-5%) of adult patients with CD diagnosed as adults, a refractory state develops despite strict adherence to a gluten-free diet (GFD).[9] In refractory coeliac disease (RCD) the number of intraepithelial lymphocytes (IELs) is markedly raised and it is from these IEL's that enteropathy associated T cell lymphoma (EATL) may arise.[9,10] Immunophenotyping of the IELs

identifies 2 groups of RCD patients: those with normal IELs (RCD I) and those with aberrant IELs, lacking surface expression of CD3 and CD8 (RCD II).[10,11] RCD II can be regarded as a cryptic lymphoma.[9] Strong molecular and immunophenotypic evidence now shows that a monoclonal neoplastic T-cell population may emerge from IEL's in RCD. Clonal expansion of this monoclonal T-cell population eventually leads to frank EATL. The genesis and expansion of these monoclonal T-cells involve both inappropriate immune responses to gluten and acquisition of genetic abnormalities. Although the monoclonal IELs in patients with RCD are neoplastic, they are not cytologically abnormal and do not form tumour masses, which differentiate these patients from EATL, in addition to the absence of radiological and bone marrow evidence of lymphoma. [10,12,13,14]

RCD II is usually resistant to any known therapy, including azathioprine/prednisone, cyclosporine and IL-10 therapy [15-18] and has a high risk of developing EATL (60%-80%, within 5 years). [10,19] This specific type of peripheral T-cell lymphomas has a very poor outcome with 1- and 5-year survival rates in the range of 31% to 39% and 11% to 20% respectively.[19,20,21] In a prospective multicenter study of 35 patients with EATL treated with 6 cycles of cyclophosphamide, doxorubicine, vincristine, prednisone (CHOP), the cumulative 2-year survival was only 28%.[11] Therefore, new treatment strategies for patients with "premalignant" CD (RCD-II) are urgently needed to improve their clinical condition with the ultimate goal of resetting the immune response, which might prevent or delay development of overt EATL.

This study reports on the feasibility, safety and efficacy of high dose chemotherapy followed by ASCT in patients with RCD II.

Patients, materials and methods

Patients

Between March 2004 and March 2006 13 patients were evaluated for ASCT. The 4 men and 3 women (mean age 61.5 years; range 51-69 years) with RCD II underwent ASCT. Six other patients were excluded because of the presence of coexistent coronary artery disease and heart failure (NYHA classification III in 2 patients), EATL found on evaluation before transplantation (3 patients), and low performance status (1 patient). One patient could not be treated due to unsuccessful leucopheresis; she developed EATL and died subsequently despite chemotherapy and immunotherapy with anti-CD52 (Alemtuzumab).[22]

The 2 patients with congestive heart failure died from progressive disease and cachexia (first patient) and bronchiectasis (second patient). The 3 patients with EATL all died within few months, whereas the patient with low performance status died from cachexia.

The baseline characteristics of the patients are shown in **table 1**. All patients received therapy with prednisone and cladribine (2-CDA) several months before receiving ASCT (not within 6 months of transplantation). The first 3 patients (patients A, B and C) were diagnosed with CD at relatively advanced age, had persistent diarrhea, weight loss and failed to respond to GFD, steroids and immunosuppressives. Because of the presence of active disease and high percentage of aberrant T cells in the small bowel

mucosa, they were included in this study protocol. At the age of 48 years, patient D was diagnosed with CD in association with dermatitis herpetiformis. Furthermore, he had a clinical picture of neurocoeliac disease with ataxia. After exclusion of structural brain and infectious disorders, he underwent ASCT at the age of 63.5 years.
Patient E has, in addition to CD with ulcerative jejunitis, Hashimoto's thyroiditis, and patient F has CD with ulcerative jejunitis. One patient (patient G) was included because of the presence of very extensive ulcerative jejunitis with multiple small bowel strictures necessating repeated resections although initially biopsies showed a low percentage of aberrant T cells. He had clinically short bowel syndrome (remaining small bowel approximately100-150 cm) requiring total parenteral nutrition (TPN).

Criteria for diagnosis of RCD

Patients with CD were considered to be refractory when symptoms of malabsorption due to villous atrophy persisted or recurred after a former good response despite strict adherence to a GFD for at least 1 year. Furthermore, possible underlying diseases such as autoimmune enteritis, bacterial overgrowth, giardiasis, amyloidosis, intestinal lymphangiectasia, Whipple's disease, hypogamma-globulinemia, eosinophilic enteritis, EATL, and inflammatory bowel disease were excluded.[11] The diagnosis of RCD was established as type II when 20% or more aberrant T cells were present.[10,11,15]

Inclusion criteria

Patients were included only when the diagnosis of true RCD with aberrant T cells was confirmed (except for patient G who was included based on the extensive ulcerative jejunitis with short bowel syndrome despite having only 10% aberrant T cells), after verifying their strict adherence to a GFD. Performance status according to the World Health Organization (WHO) criteria needed to be 0 to 2, and no severe concomitant cardiac, pulmonary, renal or hepatic disease could be present. EATL was excluded by endoscopic examination with multiple biopsies, computed tomography (CT) scan, positron emission tomography (PET), and a trephine bone marrow biopsy. Furthermore, neither active uncontrolled infection nor HIV positivity was permitted.

Evaluation

Before proceeding to ASCT, the patients were extensively evaluated as to their performance status, the presence of concomitant diseases, and extraintestinal disease or EATL. This evaluation included clinical assessment noting particularly signs and symptoms of malabsorption, body mass index (BMI) and performance according to the WHO score[23]; evaluation of adherence to a GFD including frequent consultation with dietician (advice and follow up) in addition to checking serology (anti-endomysium [EMA] and anti-tissue transglutaminase antibody [anti-tTG], both of which usually revert to negative after strict adherence to the GFD); and evaluation by upper gastrointestinal (UGIE) , video capsule endoscopy (VCE), and double balloon enteroscopy (DBE) . Duodenal biopsies (4 biopsies) were classified according to the modified Marsh criteria.[24,25] T-cell receptor (TCR-) gene rearrangement study, [12-14] T-cell flow-cytometry,

and IEL phenotyping were performed. [15,26,27] Laboratory evaluation included whole blood cell counts and serum levels of creatinine, bilirubin, liver enzymes, lactate deydrogenase, albumin, electrolytes, iron, ferritin, folic acid and vitamin B12 were determined. EMA and anti-tTG assays, HLA-DQ typing, thyroid function tests, stool examination for *Giardia* and other parasites and HIV serology were also performed.[28] For radiological evaluation, the patients underwent whole-body CT scanning and whole-body PET to exclude intestinal and extraintestinal localization of EATL. [29,30]

Immunophenotyping of IELs

IELs were isolated from 3 duodenal biopsies by passing them through nylon filters (1x100μm, 1x 40μm, BD Biosciences, Discovery Labware, Bedford, MA). Cells were stained with fluorescent-labeled monoclonal antibodies to CD3, CD7, CD8, CD45, CD103, and TCRγδ, as well as with relevant isotype controls.

All monoclonal antibodies were from BD (BD biosciences, San Jose, CA), except for CD103, which was from IQ-products (Groningen, The Netherlands) and analyzed by 4-color flow-cytometry (FACS-Calibur, BD Biosciences, San Jose, CA). Leucocyte common antigen (CD45) was always included to identify the lymphocyte population. In some tubes cell surface CD3 staining (anti-CD3-APC) was followed by permeabilization (Cytofix /Cytoperm, BD Biosciences Pharmingen, San Jose, CA) and subsequent cytoplasmic staining with anti-CD3-FITC or isotype control. Aberrant T cells were defined either as $CD7^+$ surface $CD3^-$ cells (expressed as % of CD103+ lymphocytes) or as cytoplasmic $CD3^+$, surface $CD3^-$cells (expressed as % of CD103+ lymphocytes).[12,26]

All flow-cytometry analyses were performed by an analyst and interpreted by the same medical immunologist, histopathology was performed by the same pathologist to ensure uniformity, reproducibility and consistency of results.

Assessment of TCR gene rearrangement by Polymerase Chain Reaction (PCR)

TCR-γ gene rearrangements studies were performed in separate 3 to 4 duodenal specimens that were preserved on Histocon (Polysciences Europe, Eppelheim, Germany) and frozen at –20°C. DNA was extracted from cryosections of duodenal specimens by a standard procedure using proteinase-K digestion and ethanol precipitation of the DNA. TCR-γ gene rearrangements were analyzed by multiplex polymerase chain reaction (PCR) amplification under standardized conditions. A monoclonal and polyclonal control was included in each experiment. Clonality assessment for TCR-γ gene rearrangements was done according to the Biomed-2 concerted action BM H4-CT98-3936 on PCR-based clonality studies for early diagnosis of lymphoproliferative disorders[12-14].

Peripheral blood stem cells mobilization and collection

Mobilization of hematopoietic progenitor cells from the bone marrow into the peripheral blood was achieved using granulocyte-colony stimulating factor (G-CSF) 2×5 μgm/kg by subcutaneous injection for at least 4 days. Hematopoietic stem cells were harvested from the peripheral blood by leukapheresis and kept frozen until ASCT. The target CD34+ count was more than 2×10^6/kg.

Patients	A	B	C	D	E	F	G
Age/gender	62/M	70/M	65/F	63/M	64/F	59/F	51/M
Age CD (yrs)	56	62	61	48	44	47	50
Age RCD II (yrs)	59	64	63	63	56	58	51
Age ASCT (yrs)	60	68	64	63	64	59	51
Date ASCT	March 2004	August 2004	May 2005	August 2005	Nov.2005	Dec. 2005	March 2006
HLA-DQ 2	Homozygous	Homozygous	Heterozygous	Homozygous	Heterozygous	Heterozygous	Homozygous
Marsh at RCD diagnosis	IIIA	IIIB	IIIA	IIIA	IIIC	IIIC	IIIA
BMI (Kg/m2)	19,4	18,9	17,1	24,1	20,1	21,3	20,5
Performance	1	1	1	1	1	1	2
Pred/2-CDA	Pred/2-CDA	Pred /2-CDA	Pred / 2-CDA	Pred / 2-CDA	Pred/2-CDA	Pred / 2-CDA	Pred / 2-CDA
Symptoms/ Associations	Diarrhoea, pain, weight loss	Pain, diarrhoea	Diarrhoea, Weight loss, Hypocalcemia	Diarrhoea, Weight loss, Dermatitis herpetiformis, neurological symptoms (ataxia)	Weight loss, skin rash, Hashimoto's thyroiditis	Weight loss, diarrhoea	Diarrhoea, Hypocalcemia, weight loss, extensive small bowel resection
Serology at CD diagnosis	EMA+, anti-TTG+	EMA+, Anti-TTG+	EMA+, anti-TTG+	EMA+, anti -TTG+	EMA+, anti- TTG+	EMA+, anti- TTG+	EMA+, anti- TTG+
Serology at RCD diagnosis	EMA-, anti-TTG-	EMA-, Anti-TTG-	EMA-, anti-TTG-	EMA-, anti -TTG-	EMA-, anti- TTG-	EMA-, anti- TTG-	EMA-, anti- TTG-
Endoscopy (GDS,VCE, DBE)	Nodular mucosa	Mosaic mucosa, erosions and ulcerations	Mosaic mucosa, visible vessels, no ulcerations	Nodular mucosa, disappearance of folds, erosions	Ulcerative jejunitis	Ulcerative jejunitis	Ulcerative jejunitis with multiple stenosis
CT scan	Splenic atrophy, thickened SI wall	Thickened SI loops	Splenic atrophy, dilated SI loops	Splenic atrophy	No abnormality	No abnormality	Small intestine ileus
PET scan	Increased uptake in SI	Increased uptake in SI	Increased uptake in SI	No abnormality	No abnormality	No abnormality	No abnormality

Table 1 Baseline characteristics of the patients.
M=male, F= female, Pred=prednisone, 2-CDA=Cladribine, SI= small intestine, GDS= Gastroduodenoscopy VCE= Video capsule enteroscopy, DBE= double-balloon enteroscopy

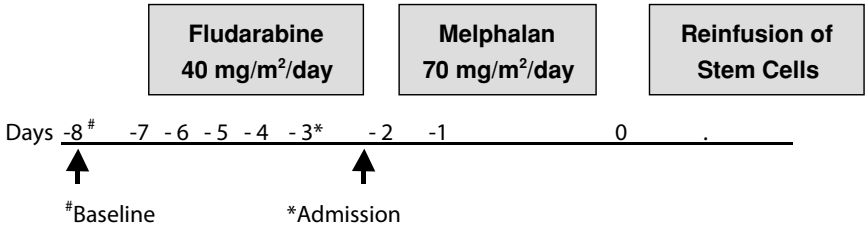


Figure 1 Scheme of transplantation protocol

Conditioning and ASCT

The conditioning regimen consisted of fludarabine given orally for 5 days (40 mg/m^2/d) and melphalan (given intravenous, 2 days 70 mg/m^2/day) as shown in **figure 1**. At day 0, the frozen stem-cell suspension was thawed and reinfused. The rationale for this conditioning regimen was based on T cell depletion by a purine analog combined with a modified dose of melphalan (total dose 140 mg/m^2) for myeloablation.

Supportive care

Patients A, C and D were supported with parenteral feeding during the 2-week period of oral mucositis after ASCT, while patient G was receiving parenteral nutritional support before receiving the transplant. After discharge, all patients except patient G were able to be fed enterally Patient G was supported to gain weight for several months with a duodenal feeding tube and limited TPN (twice a week). During admission, all patients received standard antibacterial and antifungal prophylaxis. Pneumocystis jiroveci pneumonia prophylaxis was initiated (trimethoprim-sulphamethoxazole gluten-free syrup 480-960 mg daily) until 6 months after transplantation. No patient received antidiarrheal or narcotic medications in the peritransplantation period. Blood and platelet transfusions were given as indicated.

Follow - up and criteria of response

During follow up, WHO performance, nutritional status, changes in weight and stool frequency were noted, as well as relevant biochemical markers. An endoscopic and histological examination of the small intestine was performed (3, 12 and 24 months post ASCT). From the second part of duodenum, 4 biopsies were taken for histological assessment and 4-6 for T-cell flow-cytometry study. Hematological data (hemoglobin, white blood cell [WBC] count, differential, and platelets) were registered before inclusion, after preconditioning, and after transplantation until recovery. The nadir WBC count, duration of neutropenia, infectious complications, bleeding tendency, and need for supportive therapies such as blood and platelet transfusions were documented.

Ethical approval and Informed consent

Approval of the medical ethics committee was obtained, and all treated patients signed an informed consent in accordance with the Declaration of Helsinki.

Results

Table 1 summarizes the demographic and clinical characteristics of the patients before ASCT. The mean age at diagnosis of CD was 52.5 years (range 47- 62 years) and for RCD-II 59 years (range 51-64 years). Four patients were DQ2 homozygous and 3 were heterozygous.[31] The mean follow-up was 15.5 months (range 7-30 months). All patients had a WHO performance status of 1 except patient G, whose performance status was 2. Patients B, E, F and G had ulcerative jejunitis. Patients A, C and D had splenic atrophy on CT scan. PET scan showed an increased uptake in the small intestine in patients A, B and C. At the time of diagnosis of CD, all patients were positive for anti-

Patients	A		B		C		D		E		F		G	
Duration of follow up (months)	30		27		16		8		11		10		7	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
BMI (Kg/m2)	19,4	25,6	18,9	26,1	22	24,1	24	24,1	20,1	22	22,1	23	20,5	25,8
Diarrhoea	+	-	+	-	+	+	+	-	+	-	+	-	+	-
Performance status	1	0	1	0	1	1	1	0	1	0	1	0	2	0
Albumin	32	43	33	41	30	46	32	41	20	41	30	44	26	46
Serum iron	18	26	11	18	14	10	14	15	17	13	7	17	13	18
Serum calcium	2,20	2,36	2,33	2,45	2,26	2,26	2,26	2,32	2,33	2,41	2,02	2,35	2,00	2,29
Serum folic acid	10	44	10,4	24	14	91	14	15	14	78	4,4	35	29	18
Serum B12	470	560	169	307	440	290	440	790	206	666	107	317	269	221

Table 2 Clinical and laboratory tests before and at the last follow up after ASCT. Normal range: Albumin (34-50 g/l), iron (10-32 uM0l/l), calcium (2,20-2,60 mmol/l),folic acid (>5,9 nmol/l), B12 (156-672 pMol/l

tTG and EMA, but all reverted to negative after GFD. Before and after ASCT all patients remained negative for anti-tTG and EMA.There was no transplant related mortality. The conditioning regimen seems feasible in this group of patients. The mean duration of hospitalization was 19,5 days (range 18-22 days). ASCT-related toxicity was relatively mild. Patient B had transient diarrhea and fever of undetermined origin, which was treated with intravenous antibiotics. Three weeks after discharge from the hospital, he suffered from a transient visual disturbance caused by minor retinal bleeding, which was not related to thrombocytopenia. Patient D experienced fever of undetermined origin and recovered after administration of intravenous antibiotics. One month after ASCT, patient E developed self-limiting erythematous plaque skin lesions with central necrosis. Detailed histopathological tests excluded EATL and showed aberrant T lymphocyte infiltration (CD8- CD7+ CD30+).

The mean time from the day of transplantation to neutrophil recovery was 17.8 days (range 10-21 days). Only one patient (patient B) had a transient 5-day period of severe thrombocytopenia of 5×10^9/l, all other patients had nadir platelets counts between 17 and 32 $\times10^9$/l without need for platelet transfusions.

	Marsh category		CD7+CD3- % of CD103+ ly		Cyt CD3+surf CD3- % of CD103+ ly		CD8+ % of CD103+ ly	
Patient	Before	After	Before	After	Before	After	Before	After
		3- 12- 24m		3- 12- 24m		3- 12- 24m		3- 12- 24m
A	IIIA	IIIA - IIIA - I	95	47- 48 -15	94	89 - 86 - 3	1	20- 7 - 52
B	IIIB	I - I - I	51	7 4 - 8	51	2 - 6 - 4	28	68 - 62 - 67
C	IIIC	IIIA - IIIA	62	33 - 24	59	34 - 27	15	41 - 36
D	IIIA	IIIA	54	47	81	78	7	13
E	IIIB	I	44	68	30	36	22	2
F	IIIB	IIIA	71	40	50	31	23	11
G	IIIC	IIIA	11	30	10	27	63	52
Mean	-	-	63	38	61	42	23	30

ly= lymphocytes. Normal range for Cyt CD3+surf CD3- % of lymphocytes ≤ 10%. TCRγ-PCR analysis =T cell receptor γ–polymerase chain rearrangement. Mean* = calculated for values at 3 months post ASCT.

Table 3 Histological and phenotypical flow-cytometric analysis of IEL's in duodenal biopsies before (1-6 months) and after (3, 12 and 24 months) ASCT. All patients had a clonal rearrangement of the TCRγ gene on TCRγ-PCR analysis

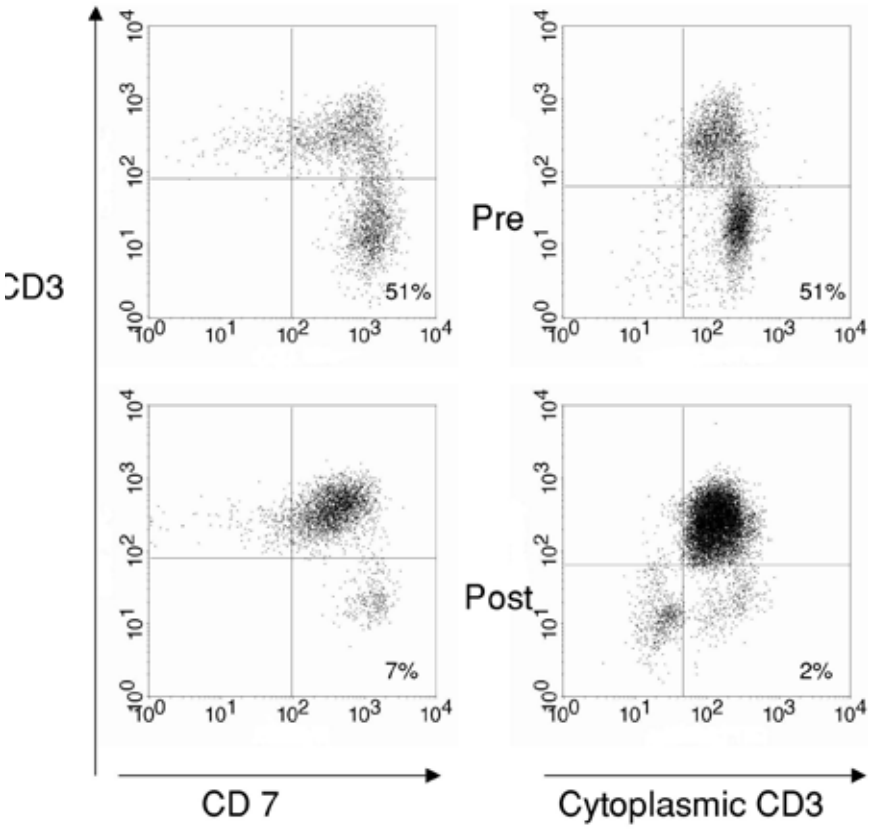
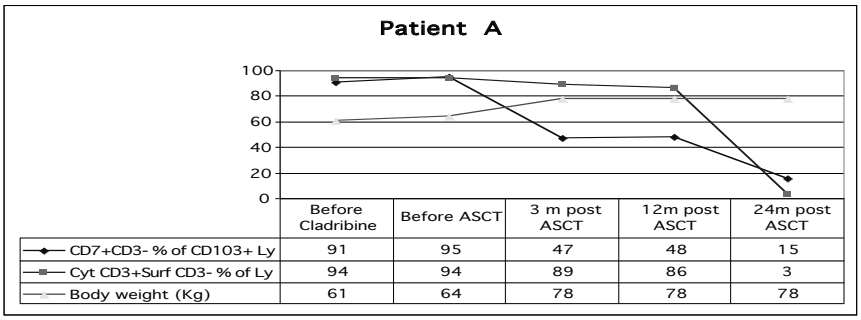


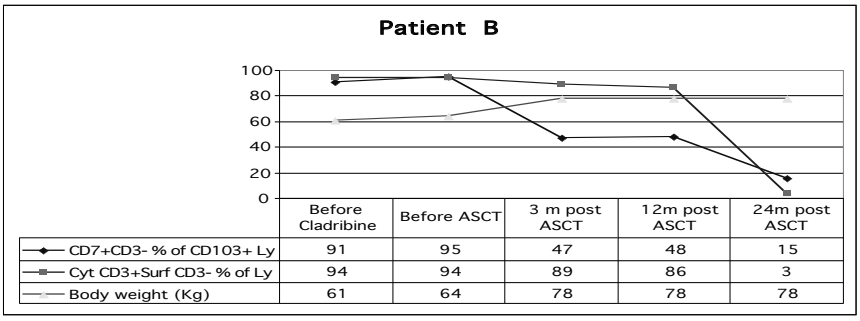
Figure 2 Flow-cytometric analysis of duodenal cells obtained from patient B, showing the change in the percentage of aberrant T-cell population pre- and post- ASCT. Aberrant population is shown as CD7+CD3- within CD103+ lymphocytes (left hand side) or as cytoplasmic CD3+ surface CD3- within lymphocyte gate (right hand side). Normal range for Cyt CD3+surf CD3- % of CD103+ lymphocytes ≤10%

Clinical and laboratory tests before and after ASCT are shown in **table 2**. Patients A, C and D were supported with parenteral feeding during the period of oral mucositis. No patient received antidiarrheal or long term narcotic medications. Within 3 months after ASCT, all patients showed impressive clinical improvement with normalization of stool frequency, disappearance of abdominal pain, and improvement of biochemical markers. In addition, improvement of BMI was documented [from mean 20,2 at baseline to 24,1 after ASCT]. Mean serum albumin level increased from 29 g/l to 40,7 g/l. Patient G showed a remarkable clinical improvement 3 to 4 months after ASCT and was able to be fed partly enterally with parenteral nutritional support twice a week.

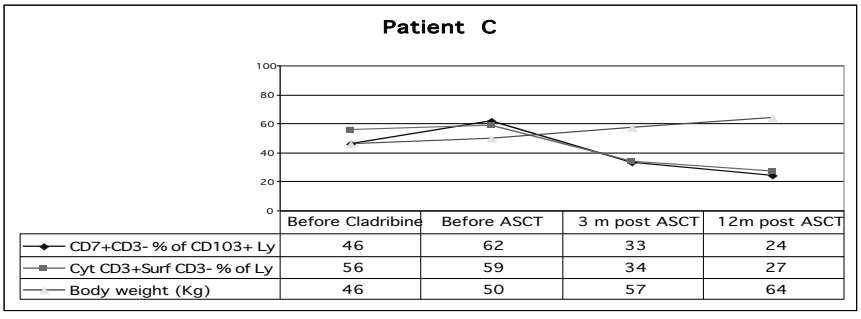
Table 3 shows the endoscopic and immunological results. All patients were monoclonal for the TCR-γ. Endoscopically there was disappearance of erosions and ulcerations in the jejunum in all patients (patients B, E, F and G) who had ulcerative jejunitis



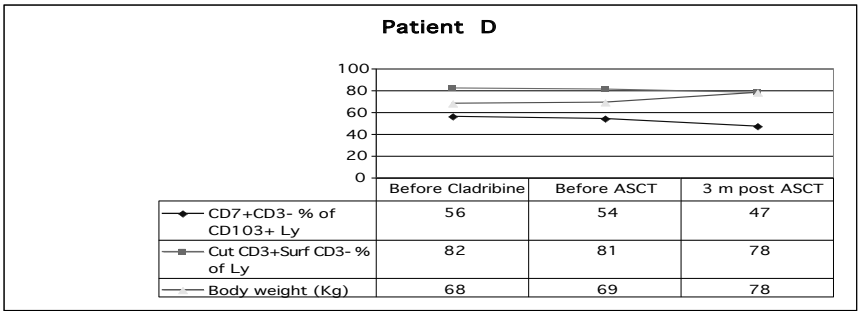
	Before Cladribine	Before ASCT	3 m post ASCT	12m post ASCT	24m post ASCT
CD7+CD3- % of CD103+ Ly	91	95	47	48	15
Cyt CD3+Surf CD3- % of Ly	94	94	89	86	3
Body weight (Kg)	61	64	78	78	78



	Before Cladribine	Before ASCT	3 m post ASCT	12m post ASCT	24m post ASCT
CD7+CD3- % of CD103+ Ly	91	95	47	48	15
Cyt CD3+Surf CD3- % of Ly	94	94	89	86	3
Body weight (Kg)	61	64	78	78	78



	Before Cladribine	Before ASCT	3 m post ASCT	12m post ASCT
CD7+CD3- % of CD103+ Ly	46	62	33	24
Cyt CD3+Surf CD3- % of Ly	56	59	34	27
Body weight (Kg)	46	50	57	64



	Before Cladribine	Before ASCT	3 m post ASCT
CD7+CD3- % of CD103+ Ly	56	54	47
Cut CD3+Surf CD3- % of Ly	82	81	78
Body weight (Kg)	68	69	78

Figure 3 The trend of aberrant T cells and body weight per patient. Ly= lymphocytes. Before = 1-3 months. Normal range for Cyt CD3+surf CD3- % of lymphocytes ≤ 10%

before ASCT, and histology of the small intestine showed significant regeneration as documented by down-staging of the Marsh class (patients A, B, C, E, F and G). Overall, the aberrant [CD7+CD3-] T-cell percentage of CD103+ lymphocytes decreased from a mean of 63% (range 11-95%) at baseline to 38% (range 7-68%) 3 to 4 months after transplantation. Aberrant cytoplasmic CD3+ surface CD3- T-cell percentage of CD103+ lymphocytes has decreased from a mean of 61% (range 10-94%) to 42% (range 2-89%). Furthermore, the mean percentage of CD8+ cells increased from 23% to 30% after ASCT. This was particularly noticeable in the first 3 patients. Patient D did not show a significant increase in CD8+ cells and the last 3 patients have not yet shown a significant change. Individual responses to ASCT differed from each patient as shown in **table 3**. Patient B showed the most impressive response with a virtual complete disappearance of aberrant T cells. The fluorescent-activated cell-sorting (FACS) data form patient B is shown in **figure 2**. The trend of aberrant T cells and body weight for the first 4 patients who have a follow- up period of at least 1 year is shown in **figure 3**. Follow-up of patients E, F and G is yet limited. Two years after transplantation, our first patient (patient A) is showing further improvement in his immunopathology status as demonstrated in further decline in the percentage of aberrant T cells to 3% and histologically improved from Marsh III-A to Marsh-I and the second patient (patient B) is still showing persistent complete clinical and histological response. Patient D had no significant change in the percentage of aberrant T cells and showed no histological improvement and also no significant improvement in CD8+ percentage; he died 8 months after transplantation. After ruling out structural and infectious (bacterial and viral) causes, we assumed that progressive disease of RCD-II with oligoclonal T lymphocytes infiltrating the brain was the cause of death in this particular patient. EATL could not be detected. Autopsy confirmed the presence of chronic encephalitis of the right temporal lobe with T-lymphocytes infiltration. Immunohistochemistry showed that the lymphocyte infiltrate was $CD3^+$ and the majority of cells expressed CD8 positivity. TCR gene analysis showed that the T- cells were oligoclonal.

Discussion

In this pilot study, ASCT in patients with RCD II patients was shown to be feasible. The conditioning regimen was well tolerated in all patients and there was a substantial clinical improvement. The rapid initial response (within 3 months) and the duration (2 years in patient A and B and 14 months in patient C) of the remission up to now are promising. Complications included the occurrence of neutropenic fever in 2 patients and retinal bleeding not related to thrombocytopenia in one patient, all with full recovery. The nadir leucocytes and platelets counts and the duration of leucopenia and thrombocytopenia were comparable to our experience in patients with Non-Hodgkin lymphomas and multiple myeloma undergoing ASCT after a combination of carmustine, etoposid, cytarabine, and melphalan (BEAM) or high dose melphalan (HDM,200 mg/m^2).[32] Because there is no standard conditioning regimen for ASCT used in autoimmune disease [33], a standard regimen from our institution was used. Fludarabine induces T cell depletion and the alkylating agent melphalan was used to achieve myeloablation.

One patient was excluded due to unsuccessful leucopheresis. Although we could achieve successful leucopheresis in all patients despite earlier 2-CDA therapy, it is possible that the reason for failure of stem cell mobilization in one particular patient might be related to the use of 2-CDA.[34] T cells play an essential role in the pathogenesis of CD and RCD-II / EATL. [8,10,15] Through the activity of the enzyme tissue transglutaminase (tTG) glutamine residues in gluten are converted into glutamic acid. [35,36] Subsequently a multitude of gluten-derived peptides is generated that, when bound to either HLA-DQ2 or DQ8 can induce T-cell responses in patients with CD. [8,24] A particular glutamine and proline-rich 33-mer α-gliadin peptide that contains 6 different T-cell stimulatory sequences and is resistant to gastric and duodenal proteolysis might be the primary initiator of the inflammatory response to gluten. In the large majority of patients, even in children with CD, inflammatory T-cell responses to other gluten peptides are also observed, implicating multiple gluten peptides in the disease process. [26,27]

The definition of RCD I/ II has undergone refinement in recent years. It seems that the most reliable available method to differentiate between RCD I and RCD II is flow-cytometry of intestinal biopsies revealing the presence of aberrant T cells. Detection of a clonal T cell population by testing for TCR rearrangement was thought to be highly predictive of EATL development. However, oligoclonal or monoclonal IELs populations can be detected in the large majority of both RCD I and RCD II patients and also in patients who do not develop an EATL. Clonality is therefore of limited use in establishing the diagnosis of RCD and to predict the development of EATL .[14,37,38]

RCD II is usually resistant to any known immunosuppressive therapy, including azathioprine/ prednisone[15], cyclosporine [16] and IL 10 therapy.[17] Recently, we treated 17 patients with 2-CDA on intention to induce remission. Within a mean follow up period of 22 months (range 7- 67 months) 47% had a significant decrease in aberrant T-cell percentages with or without clinical response.[39] However, another 41% did not respond clinically, histologically, nor immunopathologically and subsequently died from EATL. Remissions of autoimmune diseases have been described in adults after both allogenic and autologous SCT [1-7] most probably due to the extreme immunosuppressive effects of these strategies[1], resulting in immunoablation with subsequent regeneration of naïve T lymphocytes derived from reinfused hematopoietic progenitor cells.[7] Furthermore, recently, interesting insights into possible unsuspected mechanisms by which stem cell transplantation could affect the gut have emerged. In both animal and patient studies, sex mismatched allogeneic stem cell transplants have shown in both mice and women that a population of myofibroblast derived from the donor populates the intestinal mucosa. Given the importance of myofibroblasts in orchestrating the function of epithelial cells, these data suggest a mechanism other than one targeted at immunosuppression that could beneficially reset patient functions, for example, enhancing barrier function following stem cell transplantation.[40]

These positive results, the high risk of transforming into EATL and the absence of effective therapy for RCD with aberrant T-cells led us to introduce this new strategy with the ultimate goal of resetting the immune response that might prevent or delays development of overt EATL. On follow up, our patients showed improvement in the

small intestinal histology, together with impressive clinical improvement as demonstrated by disappearance of diarrhea and abdominal pain, normalization of serum albumin, electrolytes, and hemoglobin; increase in BMI; and improvement of the performance status. Two years after transplantation, our first patient is showing further improvement in his immunopathology status as demonstrated by further decline in the percentage of aberrant T cells to 3% and histologic improvement from Marsh III-A to Marsh-I. We propose that enhanced apoptosis of activated but aberrant T cells has led to this late but remarkable decline.[41] One patient died 8 months after ASCT from progressive neurological manifestations in association with CD. Autopsy had excluded any structural or infectious cause. One patient developed self-limiting erythematous plaque skin lesions with central necrosis 2 months after ASCT. Detailed analysis excluded the presence of EATL. Our most recent patient with clinically short bowel syndrome is showing remarkable clinical, endoscopical, and immunological improvement. All our patients had negative serology before inclusion, confirming their strict adherence to GFD, and after ASCT all patients remained negative for anti-tTG and EMA. Furthermore, the first 3 patients showed a significant increase in the percentage of CD8+ lymphocytes, which is seen as a marker of lymphocyte regeneration after ASCT.[42] Patient D did not show a significant increase in CD8+ cells and the last 3 patients have not yet shown a significant change. Absence of a demonstrable improvement in the surface expression of CD8 on the IEL might be regarded as a poor prognostic indicator of response; this is only to be proved or disproved on longer-term follow-up.

Although the short-term results in these patients are promising, follow-up at present is too short to permit firm conclusions as to efficacy. The selection of patients for this treatment should be restricted to those patients with a substantial population of aberrant T cells, even after therapy with 2- CDA, who have a greater tendency to progress to highly lethal EATL. High-dose chemotherapy followed by ASCT seems feasible and safe and might result in long-term improvement of disease activity in RCD patients with aberrant T cells whose condition previously did not respond to available treatments. Longer-term follow-up and additional pilot studies with larger groups of patients are needed to confirm the efficacy of this therapy.

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12. Disappointing Outcome of Autologous Stem Cell Transplantation for Enteropathy Associated T-cell Lymphoma.

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Abstract

Background: Despite treatment, enteropathy associated T-cell lymphoma has a very poor outcome. Chemotherapy can be complicated by small bowel perforation, gastrointestinal bleeding and development of enterocolic fistulae.
Here we report on the feasibility, safety and efficacy of high-dose chemotherapy followed by autologous stem cell transplantation in patients with enteropathy associated T-cell lymphoma (three upfront and one at relapse), with or without prior partial small bowel resection.
Methods: Four patients [two males, two females, mean age 65 years (range 60-69 years)] received high dose chemotherapy followed by autologous stem cell transplantation. Partial small bowel resection have been performed in three patients.
Results: All 4 patients completed the mobilization and leucopheresis procedures successfully and subsequently received conditioning chemotherapy and transplantation. Engraftment occurred in all patients. No major non-haematological toxicity or transplantation-related mortality was observed. One patient has ongoing complete remission 32 months after transplantation. Three patients died from relapse within few months after autologous stem cell transplantation.
Conclusions: Autologous stem cell transplantation for patients with EATL seems unsatisfactory. More intensive conditioning and aggressive chemotherapy with/or without targeted immunotherapy as well as allogenous stem cell transplantation need to be explored.

Introduction

Enteropathy-associated T-cell lymphoma (EATL) is a specific type of peripheral T-cell lymphoma associated with coeliac disease and it is known for its very poor outcome: 1- and 5-year survival rates in the range of 31-39% and 11-20% respectively.[1,2] In a prospective, multicentre study of 35 patients with EATL treated with six cycles of cyclophosphamide, doxorubicine, vincristine and prednisone (CHOP), the cumulative 2-year survival was only 28%. [3]
EATL is rare, except in the coeliac disease population, where the risk has been estimated to be as high as 19.2 times that of the general population.[4] The annual incidence rate of EATL has been reported to be 0.5-1 per million people in Western countries.[5]
Strict adherence to a gluten-free diet for more than 5 years has been shown to reduce the overall cancer risk, particularly EATL, in the coeliac disease group to that of the general population.[6]
EATL can present in two different clinical patterns. There are patients with well-established coeliac disease who have responded to a gluten free diet but then deteriorate because of the development of refractory coeliac disease (RCD) or EATL. In the other group, patients are not known with coeliac disease; and the diagnosis of both coeliac disease and EATL is made more or less simultaneously (*de novo* EATL). [7]

An immunophenotypically aberrant clonal intraepithelial T-cell population has been found in up to 75% of patients with RCD.[8] Clonal T-cell receptor (TCR) gene rearrangements have been found in patients with RCD without histologic evidence of lymphoma.[9-11] It remains unclear whether chronic inflammatory conditions such as coeliac disease provoke an aberrant immune response or the underlying abnormal T-cell response is already present, creating the picture of RCD.[12] Furthermore, identifying patients at risk can be difficult, as establishing the diagnosis of RCD itself takes time.[13]

In the largest case series reporting on treatment and clinical outcome in EATL, more than half of the patients could not complete treatment due to poor nutritional status and chemotherapy-induced small bowel perforations, gastrointestinal bleeding, and development of enterocolic fistulae.[14]

There have been few case studies of EATL patients treated with chemotherapy and upfront autologous stem cell transplantation (ASCT).[1, 14-18] These reports described very small groups of patients who, after reaching complete remission (CR) had a disease-free survival ranging from 0 – 64 months after ASCT. Encouraging results came from a recent report [18] describing the treatment of six patients with upfront ASCT; four patients of them remained alive in CR at 1.83-4.32 years; two had relapse.

Here we report on the feasibility, safety and efficacy of high dose chemotherapy followed by ASCT in patients with EATL (three upfront and one at relapse), with or without prior partial small bowel resection.

Patients and methods

Patients

Four patients (two males, two females) with a diagnosis of EATL received high-dose chemotherapy followed by ASCT.

Patient characteristics are summarized in **table 1**.

Patient A is a 69-yea-old female, known to have mononeuritis multiplex and Sjögren syndrome for more than 20 years. At the age of 64 years, a diagnosis of EATL and coeliac disease was established. She was treated with gluten-free diet and partial small bowel resection followed by chemotherapy, which consisted of 8 courses CHOP therapy (without Vincristine because of the presence of peripheral neuropathy). Thereafter, she remained in CR for 18 months. Subsequently she developed relapse with localization in the jejunum. A second resection was necessary, and after recovery, second line chemotherapy was initiated, consisting of dexamethasone, cytarabine and cisplatinum (DHAP, two cycles), and etoposide, ifosfamide and methotrexate (VIM, one cycle; DHAP-VIM-DHAP). This treatment was followed by high-dose chemotherapy consisting of BCNU, etoposide, cytarabine, melphalan (BEAM) and ASCT.

Patient B is a 60-year-old female who was diagnosed with *de novo* EATL localized in the mesenteric lymph nodes. She was treated with four-cycle CHOP chemotherapy and gluten-free diet. Subsequently, she received fludarabine (40 mg/m^2/day for 5 days) and melphalan (dose 70 mg/m^2 at day -2 and day -1) followed by ASCT.

Patient C is a 66-year-old male. He was admitted because of pain in the epigastric region

	Patient A	Patient B	Patient C	Patient D
Age/gender	69/F	60/F	66/M	66/M
Age CD (yrs)	64	60	65	65
Age ASCT (yrs)	66	60	66	65
DQ 2 haplotype	Homozygous	Homozygous	Heterozygous	Heterozygous
Marsh at Dx EATL	IIIA	IIIB	IIIA	IIIA
% aberrant T cells at Dx	50%	51%	30%	NA
Immunohisto-chemical type of EATL	CD3+CD8+CD30+	CD3+CD8+CD30+	CD3+CD8+CD30+	CD3+CD8-CD30+
Extraintestinal localization	Mesenteric lymph nodes	Mesenteric lymph nodes	Mesenteric lymph nodes	Hilar, Retroperitoneal, and mesenteric nodes
Bone marrow involvement	No	Yes	No	No
Endoscopy (GDS,VCE, DBE, coloscopy)	Ulcerative jejunitis	Diffuse ulcerative jejunitis	Scalloping of folds	Ulcerative jejunitis, ulcerations in colon
CT scan/ MR enteroclysis	Dilated 2nd part with abrupt narrowing of distal duodenum	Diffuse lesions in both lungs	Dilated small bowel segment with localized thickening	Mesenteric lymphadenopathy with thickened jejunum loop
FDG-PET scan	Increased activity in upper abdomen	Increased activity in right lung and neck	Increased activity in upper abdomen	Increased activity in left hilar region
CD= coeliac disease, GDS=Gastroduodenoscopy, VCE=Video capsule endoscopy, DBE=double- balloon enteroscopy				

Table 1. Patients' characteristics. At diagnosis all patients have stage IV disease and positive TTG, EMA.

and weight loss. On computed tomography (CT) scan of the abdomen, localized thickening of the small bowel wall was seen. He underwent 1 m *en bloc* resection of small bowel segment and mesentery with primary anastomosis. Histopathologically, the diagnosis of EATL was confirmed. Subsequently, he was treated with CHOP chemotherapy (eight courses in total). Because of partial response after four courses (radiologic analysis showed multiple mesenteric lymph nodes), consolidation with fludarabine and melphalan was administered, followed by ASCT.

Patient D is a 66-year-old male, known with coeliac disease and osteoporosis. He was on gluten-free diet for 1 year. Video capsule enteroscopy (VCE) and double-balloon enteroscopy (DBE) were performed because of persistent weight loss. Both these methods showed ulcerative jejunitis of the distal jejunum and ileum, but histopathology of endoscopic biopsies was not conclusive. An emergency laparotomy was performed because of persistent melena and hemodynamic instability. Partial resection of small intestine was performed. In the resection specimen, multiple localisations of a lymphoma had been identified in the wall of the ileum.
After recovery from laparotomy, he was treated with eight-cycle CHOP chemotherapy, combined with immunotherapy (antiCD52, alemtuzumab). Subsequently, he received BEAM, followed by ASCT.

Staging

The Ann Arbor staging system was used based on clinical assessment, chest X-ray, whole body CT-scan, positron emission tomography (FDG-PET) [19], magnetic resonance (MR) enteroclysis, evaluation by an ear nose throat surgeon, and bone marrow trephine biopsy.

Criteria of diagnosis

The diagnosis of EATL was established according to the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. [20] The immunohistochemical features of EATL are the presence of large or medium-sized T-cell proliferation expressing a $CD3^+$ $CD8^{+/-}$ and $CD103^+$.
EATL can be $CD3^+$ $CD8^-$ $CD30^+$ large cell lymphomas, $CD3^+$ $CD8^+$ $CD30^-$ small cell lymphomas or γδ- lymphomas. Diagnosis of EATL was confirmed by an expert- panel of pathologists.

Peripheral blood stem cells mobilization and collection

Mobilization of haematopoietic progenitor cells from the bone marrow into the peripheral blood was performed using granulocyte-colony stimulating factor (G-CSF).
Haematopoietic stem cells were collected from the peripheral blood by leucopheresis.

Response criteria

Response to therapy was evaluated according to the Cheson criteria.[21] These criteria include anatomic definitions of response.
A *complete response* requires the following:

1 • Complete disappearance of all detectable clinical and radiographic evidence of disease and disappearance of all disease-related symptoms if present before therapy, and normalization of those biochemical abnormalities (e.g. lactate dehydrogenase) definitely assignable to NHL.
2 • All lymph nodes and nodal masses must have regressed to normal size.
3 • The spleen must have regressed in size and must not be palpable on physical examination.

4 • Bone marrow, if positive at baseline, must be histologically negative for lymphoma.
Complete Response, unconfirmed (CRu) includes those patients who fulfil criteria 1 and 3 above, but with one or more of the following features:

- A residual lymph node mass greater than 1.5 cm in greatest transverse diameter that has regressed by more than 75% in the size. Individual nodes that were previously confluent must have regressed by more than 75% in their size compared with the size of the original mass.
- Indeterminate bone marrow (increased number or size of aggregates without cytologic or architectural atypia).

A partial response (PR) requires the following:

- More than 50% decrease in size of the six largest dominant nodes or nodal masses.
- No increase in the size of the other nodes, liver, or spleen
- Splenic and hepatic nodules must regress by at least 50% in the size.
- With the exception of splenic and hepatic nodules, involvement of other organs is considered assessable and not measurable disease.
- No new sites of disease

Stable disease is defined as less than a PR (as described above) but not progressive disease (see below).

Progressive Disease is defined as 50% increase from the nadir in the size of any previously identified abnormal node or the appearance of any new lesion during or at the end of therapy.

Results

Patient characteristics

The baseline characteristics of the four patients are shown in **table 1**. The mean age 65 years (range 60-69 years).Three patients had ***de novo*** type of EATL, while one patient was known to have coeliac disease 1 year before developing EATL (patient D). All patients had positive serology for coeliac disease at diagnosis.

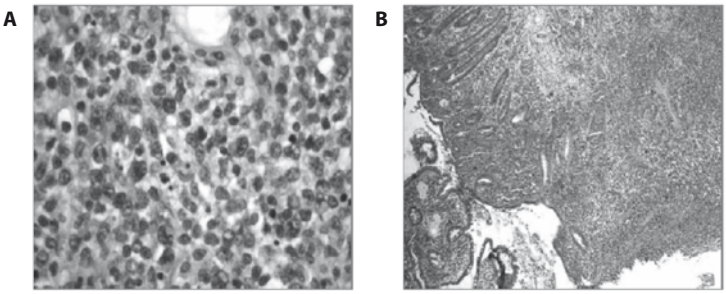

Figure 1. EATL in ileum resection specimen with a proliferation of middle-sized atypical lymphocytes (A) leading to villous atrophy and ulceration (B). (H&E, 12,5x and 630x)

Endoscopical, histological and immunophenotypical features

Endoscopically, three patients had evidence of ulcerative jejunitis. All patients had definite histological features of coeliac disease according to the Marsh criteria (three had Marsh IIIA and one IIIB). A significant percentage (30-51%) of the intraepithelial lymhocytes was aberrant, defined as $CD7^+$ surface $CD3^-$ cells (expressed as percent of $CD103^+$ lymphocytes) or cytoplasmic $CD3^+$, surface CD3 negative cells (expressed as percent of $CD103^+$ lymphocytes) in all patients.

Immunophenotypical testing showed that these malignanT-cells were $CD3^+CD8^-CD30^+$ in 3 patients (patients A, B and C) and $CD3^+CD8^+CD30^-$ in patient (patient D).

The histological and immunophenotypical features of patient D are shown in **figures 1 and 2**, respectively.

Stem cell mobilization

Mobilization of haematopoietic progenitor cells from peripheral blood was achieved successfully and uncomplicated in all patients using G-CSF.

Transplantation-related toxicity

There was no transplantation-related mortality or serious morbidity. The mean duration of hospitalization was 20 days (range 18-24 days). Haematopoietic recovery was fast in all patients. The median time to reach neutrophils > $0,5\times10^6$/l and unsupported platelets > 20×10^6/l were 12 days (8-15) and 14 days (9-16), respectively.

Response and survival after ASCT

Table 2 summarizes the responses to treatments. One patient (patient A) was in remission for 18 months after standard chemotherapy and received ASCT in second CR. She is in ongoing CR at 32 months.

Patient B developed severe neurological complaints at 4 weeks after ASCT before response to transplantation could be assessed. A cauda equina syndrome was diagnosed. CSF examination confirmed the presence of lymphoma cells carrying the same immunological markers ($CD3^+CD8^+CD30^+$). She received palliative radiotherapy. Unfortunately, she died from this rapidly progressing CNS relapse 2 months after ASCT.

Patient C was admitted 6 months after ASCT because of persistent gastrointestinal bleeding and pancytopenia. He had partial remission after chemotherapy and subsequently CR after ASCT. He received supportive care (blood and platelet transfusions). Relapsed EATL was suspected, but all supportive care was withdrawn after explicit request of the patient and his family, and he died several days later. Autopsy examination showed the presence of a large amount of blood in the gastrointestinal tract and multiple mesenteric pathological lymph nodes (**Figure 3**). Microscopic examination of these nodes confirmed the presence of EATL in relapse.

Patient D had shown initial CR after chemotherapy and remained so after ASCT, but he developed a relapse 9 months after transplantation. He was admitted with bleeding per rectum. Colonoscopy showed multiple deep ulcerations in the ascending colon and terminal ileum. Histopathological examination confirmed EATL relapse. He died

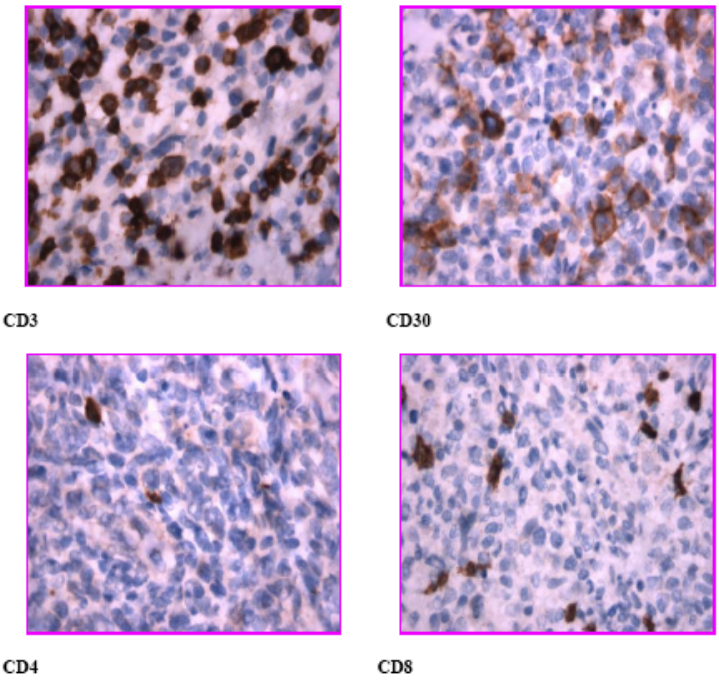

Figure 2. EATL in ileum resection specimen expressing CD3 and partly also CD30. Residual small T lymphocytes express CD4 or CD8, lymphoma cells virtually all negative.

Patients	A	B	C	D
Follow up (ms) after SCT	32	2	6	9
Resection performed	Yes	No	Yes	Yes
Response to CHOP	CR (18 m) followed by relapse	CR	PR	CR
Other therapies	DHAP-VIM-DHAP	-	-	Alemtuzumab
Preconditioning regimen	BEAM	Flu+Mel	Flu+Mel	BEAM
Relapse after ASCT	No	Yes	Yes	Yes

Table 2. Treatment results. CR= complete remission, PR=partial remission, Flu+Mel= fludarabine and melphalan

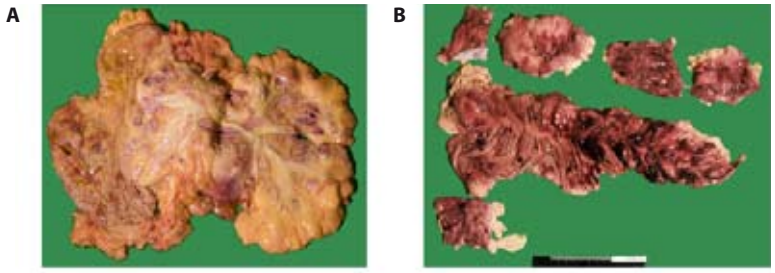

Figure 3. Macroscopic picture showing multiple pathological mesenteric lymph nodes (A) and large amount of blood in the small intestine (B)

and an autopsy examination showed the presence of a large amount of blood in the gastrointestinal tract due to numerous ulcers in the small intestine and the colon with multiple mesenteric pathological lymph nodes. Microscopic examination of these nodes showed considerable depletion of lymphocytes, but the pathological lymphoid population could not be identified with certainty in the lymph nodes.

Discussion

Intestinal T-cell lymphomas or EATLs respond poorly to available anti-lymphoma regimens. Results are better in patients with limited stages of disease.[22] The most frequent complications of treatment are small bowel perforation, obstruction, gastrointestinal bleeding, and infection.

We report here our results of the treatment of four patients with an advanced stage of EATL diagnosis (stage IV). The induction chemotherapy and "debulking" were feasible, as the patients were in good condition before undergoing ASCT. The conditioning regimen and ASCT were well tolerated in all patients. The nadir leucocytes and platelets counts and the duration of leucopenia and thrombocytopenia were comparable to that in patients with other types of non-Hodgkin lymphomas and multiple myeloma receiving ASCT after BEAM or high dose melphalan.[23]

There was no transplantation-related mortality. Our first patient received ASCT at second complete response, while the other three received upfront ASCT. These three patients have early response (two have CR), but all of them developed relapse and died.

Variable results were published dealing with ASCT in EATL and these are summarized in **table 3**. Our results are consistent with that of others. [1,14,16] Encouraging results came from a recent report by Bishton and Haynes,[18] that describes the treatment of six patients with ASCT after receiving two cycles of ifosphamide, etoposide, epirubicin (IVE), followed by two cycles of high-dose methotrexate (3 g/m^2) with folinic acid rescue and carmustine, etoposide, cytarabine, melphalan (BEAM). Four patients remain alive in CR at 1.83-4.32 years; two had relapse. However, it is important to recognise that Bishton *et al.* treated patients at an early stage of disease (40-59 years old); five of them have stage I and one patient had stage II at inclusion, in contrast to our patients who were both older (60-69

Author/ reference	No. EATL patients	No. received ASCT	High dose chemotherapy	Preconditioning	Overall survival	Comments
Gale et al [1]	31	1	PEACE-BOM	BEAM	CR 64 m	overwhelming sepsis after SCT
Okuda et al [14]	1	1	8 cycles CHOP. AI relapse ESHAP (etoposide, methyl-prednisolone, cytarabine and cisplatin.	MCVC (ranimus-tine carboplatin, etoposide & cyclophospha-mide)	8 months	Died after developing relapse (intestine and CNS)
Rongey et al [15]	1	1	4 cycles (Cyclophosphamide, Doxorubicine & Etoposide) and then 3 cycles CHOP	BEAM	CR18 m	EATL after having coeliac disease and follows gluten free diet irregularly
Jantunen et al [16]	5	5	CHOP	BEAC 3 patients/ BEAM 2 patients	median survival 2 ms (0-14m)	4 treated initially with partial small intestine resection
Blystad et al [17]	2 (total 4 0 NHL*)	2	Specific details over these 2 patients are not available	-	-	-
Bishton et al [18]	6	6	2 cycles of IVE (ifosphamide, etoposide, epirubicin), followed by two cycles of methotrexate (3 g/m2)+folinic acid rescue	BEAM	Four in CR at 1.83-4.32 yrs	2 relapsed

Table 3. Summarizes the earlier reports on ASCT in EATL. *NHL= Non-Hodgkin's lymphoma

years) and had advanced stage of disease (stage IV). Another important difference was the use of more aggressive chemotherapy by Bishton et al. The additive value of gut sterilisation before ASCT is difficult to interpret because of the lack of comparative data. Cytoreductive therapy, using chemotherapy and partial small bowel resection, seems logical. We have recognized that the patients' condition improves before chemotherapy and also prevents the occurrence of complications as perforations, fistulas and bleeding.

Intervention at an earlier stage in the evolvement of lymphoma at the pre-malignant phase (RCD with high percentage of aberrant T-cells) could theoretically prevent or delay the development of the malignant phase. Recently we reported on our experience in treating RCD patients with high-dose chemotherapy followed by ASCT, and the results thus far are promising in five of the seven transplanted patients.[24] A possible explanation might be that transplantation may eliminate the aberrant T-cell clone in RCD patients; this is in contrast to patients with EATL, who already have developed a neoplastic clone.

It is a well known that T-cell malignancies do not respond adequately to conventional chemotherapeutic treatment.[25,26] The introduction of monoclonal antibodies for the treatment of cancer may change the outlook for patients with T-cell malignancies.[27] Recent studies with single-agent alemtuzumab, an anti-CD52 monoclonal antibody, have shown some improvement of response rates and survival in patients with T-cell prolymphocytic leukaemia and cutaneous T-cell lymphoma.[28] Preliminary data also suggest that alemtuzumab may have activity in patients with heavily pre-treated peripheral T-cell lymphoma who are refractory to conventional chemotherapy.[29] Preclinical studies with mice bearing human adult T-cell leukaemia/lymphoma cells suggest that alemtuzumab may have a role in this setting.[30] Therefore, treatment of EATL with alemtuzumab in combination with chemotherapy should be explored.

Furthermore, it has been shown that patient who undergo allogeneic SCT for NHL, both indolent and high grade types, have lower relapse rates than those who undergo autologous SCT.[31, 32, 33]

It seems that current chemotherapy and high-dose conditioning regimens followed by ASCT do not improve the survival in this type of aggressive lymphoma. Relapse regularly occurs within weeks to months after ASCT. Therefore, instituting therapy at an earlier stage, the development of more effective treatments including anti-CD52 agents, better pre-conditioning regimens and possibly the use of T-cell-depleted grafts or allogeneic stem cell transplantation with or without primary central nervous system prophylaxis are urgently required to improve the prospects of these patients.

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Part five

Summary, discussion and future perspectives

Summary, general discussion and future perspectives

The current thesis reports on novel developments in diagnosis and treatment, as well as novel insights into the pathogenesis of Refractory Coeliac Disease (RCD). RCD may now be recognized as a relevant clinical entity, associated with a poor prognosis in a specific subgroup of patients. A key role for flow cytometry of intestinal lymphocyte populations has emerged, allowing accurate discrimination between both RCD subgroups. Given the association of aberrant intestinal T lymphocytes with the development of Enteropathy-Associated T-cell Lymphoma (EATL), the therapeutic challenge in RCD II is effective elimination of these cells, either chemotherapeutically or by means of stem cell transplantation. Although the underlying mechanisms of RCD development remain ill defined and require further investigation, key players have been indicated at cellular, molecular and genetic levels involved in intestinal homeostasis. The individual contributions described in this thesis relevant to the above mentioned novel insights and developments will be summarized and discussed below.

Chapter 1: Overview of RCD

Over the past 2 decades major advances have been accomplished in the field of RCD. This first chapter provides and extensive overview of RCD pathogenesis, involving genetic factors and immunological mechanisms. In addition, the work-up in establishing the diagnosis of RCD is postulated. In the distinction between RCD I and II, intraepithelial lymphocytes (IELs) play a key role. Flow cytometry and clonality analysis represent the diagnostic parameters required to make this distinction, which has important implications for prognosis and subsequent treatment. An outline of the therapeutic options thusfar reported for RCD as well as EATL patients is provided at the end of this chapter.

Chapter 2: Survival in RCD and EATL

Chapter 2 provides further insight into the prognosis of RCD and the development of EATL, by reporting on long-term survival and risk of transition of RCD into EATL in a large cohort of patients with complicated coeliac disease.

When normal expression of T-cell surface markers is present in RCD patients (type I), the prognosis appears to be less dismal than when an aberrant IEL population is present (RCD type II); 50-60% of the latter patients develops EATL within 4-6 years, after which the 5-year survival is only 8-20%. Furthermore, patients with EATL may present in two different clinical patterns. Firstly, patients with well-established coeliac disease who deteriorated because of the development of RCD II and eventually progressed to secondary EATL. In the other group patients develop EATL without a preceding history of complicated coeliac disease, often presenting with perforation or obstruction (primary or de novo EATL). Interestingly, no CD-related mortality was recognized in the RCD I group and none of our RCD I patients has progressed to RCD II during follow-up. More aggressive and targeted therapies seem mandatory in RCD II and EATL.

Chapter 3: Incidence of EATL

The third chapter provides the epidemiology of EATL in the Netherlands, by studying the incidence of EATL as well as the demographic characteristics of patients with EATL by searching the nation-wide network and registry of histo- and cyto-pathology reports in the Netherlands (abbreviated as PALGA). Clinico-pathological data were obtained for 116 cases of EATL, diagnosed between 2000 – 2006. EATL appeared to be a rare disease with a crude incidence of 0.10 per 100.000 inhabitants per year; and an incidence of 2.08/100,000 over 50 years of age, with a peak incidence in the 7th decade. The tumour was mainly localised in the proximal small intestine. Although uncomplicated CD is twice as frequent in female patients, EATL was more prevalent in males.

Chapter 4: Flow cytometry in the diagnosis of RCD

Early detection of those patients actually at risk to develop EATL is of utmost importance for curative intervention. A cut-off point between acceptably normal and pathologically increased percentages of aberrant T-cells in RCD is defined and validated in this chapter. This reference range now allows accurate distinction between RCD types I and II, having a very different prognosis as described earlier in Chapter 2. To establish an optimal cut-off value for this percentage, reference ranges for aberrant T-cells in the duodenal mucosa of different CD patient and control groups were generated. Furthermore, the predictive value of this cut-off was compared with intestinal T-cell clonality, as prognostic parameter for EATL development in RCD. Quantification of aberrant T-cells by flow cytometry was preferable to T-cell clonality analysis for identification of RCD patients at risk for EATL development. A cut-off value of 20% appeared reliable for risk stratification, therapeutic options and subsequent follow-up of RCD patients. Interestingly, the aberrant T-cells in primary EATL patients as well as ulcerative jejunitis appeared to be largely confined to tumour mass and ulcerations and could not be found in such high percentages diffusely throughout the small intestine as in RCD II and secondary EATL patients. The latter may suggest a differential pathogenesis, requiring further investigation.

Chapter 5: RCD is a disseminated disease

Chapter 5 describes whether aberrant T-lymphocytes in RCD II could be detected in other parts of the small intestinal mucosa besides the intraepithelial compartment. Additionally, the presence of aberrant T-lymphocytes was analyzed in two RCD II patients that developed ill-defined skin lesions. Multiparameter flow cytometric immunophenotyping was performed on both IEL and lamina propria lymphocyte (LPL) cell suspensions, isolated from fresh small bowel biopsy specimens of RCD II.

In RCD II the aberrant T-lymphocytes may also reside in the extraepithelial layer of the small intestinal mucosa, and even in extraintestinal localizations including the skin. Whether this phenomenon represents a passive overflow from the intestinal epithelium or active trafficking towards other anatomical localizations remains to be elucidated. RCD II appears to be a disseminated disease, which may impose the risk of EATL development outside the intestine.

Chapter 6: Aberrant IELs at the molecular level in RCD

Aberrant IELs and EATL cells are typically cytCD3+, but lack expression of the T-cell receptor (TCR)-CD3 complex on the cell surface. It is currently unknown what causes the loss of surface TCR-CD3 expression. In this chapter we report on the generation and molecular characterization of a IEL cell line, derived from a RCD II patient, with the characteristic immunophenotype of EATL. This study provides the first evidence that loss of TCR-CD3 surface expression on IELs in RCD II is due to defects in the synthesis and/or assembly of T-cell receptor chains providing a first step in understanding the process leading to the development of RCD II and subsequent progression to EATL.

Chapter 7: Do TCRγδ+ IEL protect against EATL?

TCRγδ+ IELs play an important role in mucosal repair, homeostasis and tumor surveillance. Recently, human small intestinal TCRγδ+ IELs were shown to have regulatory potential in uncomplicated CD. In Chapter 7 we investigated whether TCRγδ+ IELs are decreased in RCD II, providing a possible explanation for persisting mucosal damage and inflammation, and the emergence of aberrant T-cells with clonal expansion to EATL. A significantly lower percentage of TCRγδ+ IELs was found in RCD II as compared to all other CD groups. Overall, there was a clear negative correlation between the presence of TCRγδ+ IELs and aberrant IELs. This may imply that TCRγδ+ IELs play a crucial role in the disease process. Interestingly, TCRγδ+ IELs increased again in RCD II after therapy aimed at elimination of aberrant IELs. These cells could be important markers in flow cytometric analyses, in addition to aberrant T-cells, to differentiate between disease categories and to evaluate the effectiveness of therapeutic strategies.

Chapter 8: The role of peripheral regulatory cells in RCD

In this chapter, we investigated whether a lack of circulating homeostatic T-cells, such as Treg, Tγδ or iNKT cells would be associated with the development of RCD or EATL. In summary, our study demonstrates that only the iNKT cell numbers are selectively reduced in RCD I and II. With respect to other circulating T-cells with regulatory potential, including Treg and Tγδ cells, we did not find unusual levels, neither in responsive nor in refractory CD.

CD patients treated with a gluten free diet (GFD) displayed a significantly increased fraction of CD4+ iNKT cells. This indicates that regulatory cell numbers can increase during a GFD, or that individuals with higher frequencies of regulatory cells are more likely to respond to a GFD. Follow up studies are necessary to determine whether CD4+ iNKT cells control the immune response against gluten and if their absence contributes to the progression to RCD and EATL.

Chapter 9: Genetic predisposition in RCD

Genes play a key role in the pathogenesis of CD. The class II human leucocyte antigen (HLA)-DQ2 and HLA-DQ8 loci are the most important genetic contributors identified so far. Furthermore, uncomplicated CD has been linked to genetic variants in the MYO9B gene on chromosome 19. HLA-DQ2 homozygosity is associated with compli-

cations of CD such as RCD II and EATL. We investigated whether certain MYO9B variants also predispose to RCD II and EATL.
One single nucleotide polymorphism (SNP) in MYO9B showed a significantly different allele distribution in RCD II and EATL patients compared to controls (p=0.00002). The rs7259292 T allele was significantly more frequent in RCD II and EATL patients compared to CD patients. The frequency of the haplotype carrying the minor allele of this SNP was significantly increased in RCD II and EATL patients (11%), compared to controls (2%) and CD patients (3%). Both MYO9B rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to CD patients without evidence for interaction between these two risk factors. This study shows that both MYO9B variants and HLA-DQ2 homozygosity might contribute to a complicated course of CD.

Chapter 10: Targeted treatment of RCD?
This chapter describes the treatment of a RCD II patient with Alemtuzumab (Campath®, monoclonal anti-CD52). CD52 is expressed by all T- and B-cells and targeting of CD52 by antibodies has proven to be successful in eradicating overt lymphoma. Although the patient showed a clinical response as illustrated by an increase in bodyweight and decrease in diarrhea, the mucosal lesions persisted and the intestinal aberrant IELs even increased from 60% to 91%. Eventually this patient developed EATL. A possible explanation may be that IELs are not sufficiently reached and/or targeted by alemtuzumab, given the fact that in our patient virtually all aberrant T-cells in the intestinal mucosa still expressed CD52, whereas in peripheral blood barely any B- and T-cells could be detected.

Chapter 11: ASCT in RCD – future promise
Autologous Hematopoietic Stem Cell Transplantation (ASCT) is an increasingly accepted treatment for refractory autoimmune diseases. This study reports on the feasibility, safety and efficacy of ASCT in RCD type II, with the ultimate goal of resetting the immune response to prevent or delay development of overt EATL.
Seven RCD II patients were transplanted after conditioning with fludarabine and melphalan. Engraftment occurred in all patients. No major non-hematological toxicity or transplantation-related mortality was observed. There was a significant reduction in the amount of aberrant T-cells in duodenal biopsies associated with clinical improvement, and normalization of hematological and biochemical markers (mean follow-up 15.5 months, range 7-30 months). One patient died 8 months post-transplant from progressive neuroCD. High-dose chemotherapy followed by ASCT seems feasible and safe, and may result in long-term improvement of disease activity in RCD patients with aberrant T-cells not responsive to available treatments. However, extended follow up and additional pilot studies with larger groups of patients are needed to confirm the efficacy of this therapy.

Chapter 12: ASCT in EATL – yet to be successful
Despite treatment, EATL has a very poor outcome, as described in Chapter 2. In this study we report on the feasibility, safety and efficacy of high dose chemotherapy

followed by ASCT in 4 patients with EATL (3 upfront and 1 at relapse), with or without prior partial small bowel resection. One patient had ongoing complete remission uptill 32 months after transplantation. Three patients died from relapse within few months after transplantation. ASCT for patients with EATL seems unsatisfactory. More intensive conditioning and aggressive chemotherapy with/or without targeted immunotherapy as well as allogeneic SCT should be explored.

Future perspectives

Although the diagnostic work-up of RCD has evolved considerably, at least in part as a result of the implementation of flow cytometry, further identification of prognostic parameters for EATL development appears required. Since approximately half of the RCD II patients actually develops EATL, the mere presence of increased numbers of aberrant intestinal T-cells, and decreased numbers of intraepithelial TCRγδ+ cells, may only partly predict the development of overt lymphoma. The search for novel prognostic parameters, based on for instance specific immunophenotypic (de)differentiation of aberrant T-cells, needs to be continued. Differentiation within the RCD II patient group, between those patients that will and those that will not develop EATL, represents an ultimate target. Applying flow cytometric analysis of molecules associated with proliferative capacity (Ki-67, IL-15Rα), monitoring of further dedifferentiation (loss of CD7, CD52, CD103) and monitoring of the acquisition of EATL associated phenotype (gain of CD30) are included in current and future investigation. In addition, analysis of cytokine production profiles as well as evaluation of apoptosis resistance profiles may provide a significant contribution. Importantly, further increase in the awareness of complicated CD, with respect to both incidence and clinical presentation, is warranted.

Sofar, already a substantial amount of insight into the pathogenesis of RCD has been acquired. The impact of genetic factors involved in intestinal permeability has been recognized. At the molecular level, the underlying mechanism of defective TCR/CD3 expression by aberrant IEL has been elucidated. It is to be expected that further analysis of RCD patients at the genetic and molecular level will shed additional light onto the pathogenesis of RCD. Interestingly, T-cells with regulatory potential, including iNKT and TCRγδ+ cells, appear to be involved in the RCD disease process, both systemically and in situ. Further dissection of such regulatory cells, including in situ analysis of FoxP3+ regulatory T-cells in the small intestinal mucosa, will be of interest. In addition, the potential involvement of the recently identified pro-inflammatory Th17 subset should be considered and will open up novel research areas. Th17 cells have been identified as key players in the pathogenesis of the inflammatory bowel disease M.Crohn, an entity with obvious analogies to RCD, both being a chronic inflammatory condition.

The identification of aberrant T-cells outside the intestinal epithelial layer, and even outside the gut itself, is an intriguing finding. Whether it represents passive overflow from the epithelium, or active dissemination is currently unknown. However, it could impose a serious threat with regard to the appearance of EATL in diverse anatomical localizations. Mapping the expression profiles of different adhesion molecules and

homing receptors of aberrant IEL and/or EATL cells, presenting outside the intestine could help to predict disseminating potential of aberrant IEL still residing in the epithelial compartment.

The treatment of RCD patients remains a challenge, especially in case an aberrant IEL population is present (RCD II). The RCD I patients respond well to general immunosuppression (Azathioprine and Prednisone) and do not seem to be at risk for EATL or RCD II. However, currently there is no established treatment available for the RCD II (and UJ) patients aimed at prevention of EATL. Fortunately, therapeutic strategies are evolving rapidly. Therapies directed at eradicating the aberrant IEL population, such as upfront Cladribine, are promising. In addition, the combination with ASCT should be further explored, as these treatments have proved to significantly reduce number of aberrant IELs. In view of the increasingly successful application of monoclonal antibody based therapies, targeting of aberrant intestinal T-cells by anti-CD52 (Alemtuzumab, Campath®) was explored. However, single-agent therapy with Alemtuzumab did not appear to sufficiently eradicate aberrant T-cells and thus failed to be effective in RCD II. Its use in combination with chemotherapy and ASCT, may be still promising. Regarding future treatment of RCD II / UJ patients, optimization of therapy may imply fine-tuning the ASCT-protocol by using T-cell depleted grafts and exploring novel preconditioning regimens. Importantly, to attain a significant impact on improving the dismal prognosis of these patients (which now have a 5-year survival of 58%), multi-center collaboration and pooling of data are mandatory.

Despite treatment with combination chemotherapy and high dose conditioning regimens followed by ASCT, the survival of EATL remains very poor. Results of ASCT for EATL are unsatisfactory sofar as patients often present with an advanced stage of disease and relapse occurs regularly. Therefore, instituting therapy at an earlier stage (if possible RCD II / UJ), development of more effective combined/targeted treatments, and improved conditioning regimens are needed. More importantly, the use of allogeneic SCT with reduced intensity conditioning may hold considerable promise in these EATL patients and should be further explored. If patients are not suitable for allogeneic SCT, new options, such as a so-called "Sandwich-treatment" with Cladribine, CHOP, Cladribine (C,CHOP,C) may have a future role. To facilitate an optimal international collaboration, in order to set up multicenter trials for the treatment of RCD II / UJ and EATL patients, diagnostic criteria for these disease entities should be determined in a similar way to that done for CD in 2001. It is anticipated that efforts for this purpose will take place at the next International Coeliac Disease Symposium in Amsterdam 6-8 April 2009.

Samenvatting voor niet-ingewijden

Coeliakie is een veel voorkomende ziekte die wordt veroorzaakt door een overgevoeligheid voor gluten. Gluten is een belangrijk bestanddeel van o.a. tarwe en wordt in veel voedingsmiddelen verwerkt. Bij het eten van gluten krijgen de meeste patiënten een ontstekingsreactie in de darm en beschadigt de darmwand, hierdoor ontstaan vaak buikklachten, diarree en gewichtsverlies. De ontstekingsreactie in de darmwand wordt veroorzaakt door bepaalde cellen in de darm die verantwoordelijk zijn voor de afweer, genaamd T-lymfocyten. De laatste jaren worden er, mede dankzij de verbeterde diagnostiek, meer en meer coeliakiepatiënten gediagnosticeerd bij wie maag- darmklachten niet op de voorgrond staan. Het betreft hier patiënten met o.m. groei-achterstand, bloedarmoede, chronische vermoeidheid, hormonale stoornissen of osteoporose.
De therapie van coeliakie bestaat uit het instellen van een glutenvrij dieet, dat levenslang gevolgd moet worden. Bij vrijwel alle coeliakiepatiënten normaliseren de klachten dan en herstelt de darm. Bij een zeer gering aantal patiënten echter, veelal op volwassen leeftijd gediagnosticeerd, herstelt de darm niet en blijft de ontstekingsreactie in de darm bestaan. Er kunnen complicaties optreden, ook al volgen de patiënten een strikt glutenvrij dieet. Bij deze mensen spreekt men van refractaire coeliakie (RCD).
Een zeer ernstige complicatie van refractaire coeliakie is een bepaalde vorm van lymfeklierkanker in de dunne darm, enteropathie geassocieerd T-cel lymfoom (EATL) genoemd. Deze vorm van kanker ontstaat uit een deel van de hierbovengenoemde T-lymfocyten. Deze specifieke T-lymfocyten kenmerken zich door een afwijkend eiwitpatroon op het celoppervlak. Een EATL (enteropathie geassocieerd T-cel lymfoom) wordt vrijwel uitsluitend gevonden bij patiënten bij wie op volwassen leeftijd coeliakie werd vastgesteld, meestal voorafgegaan door een stadium van RCD (refractaire coeliakie). Op dit moment kan niet goed voorspeld worden welke RCD patiënten EATL zullen ontwikkelen en welke niet.
In dit proefschrift worden de ontwikkelingen op het gebied van de diagnose en de daaropvolgende behandeling van RCD en EATL besproken, daarnaast wordt inzicht gegeven in factoren die een rol spelen in het ontstaan van RCD en EATL. Er wordt onderscheid gemaakt in 2 verschillende typen RCD patiënten, type I en type II, waarvan alleen type II het risico heeft op het ontwikkelen van een lymfoom. Een duidelijk criterium om onderscheid te maken tussen deze 2 subtypen ontbrak nog in de literatuur. Alleen het 'aanwezig zijn' van de hierboven genoemde afwijkende T-cellen in de dunne darm (type II) zou mogelijk een voorspellende waarde hebben bij het uiteindelijk ontwikkelen van EATL. Zoals al eerder gezegd hebben deze T-cellen een afwijkend ofwel 'aberrant' eiwitpatroon aan het oppervlakte, ook wel celoppervlakte 'markers' genoemd, aan de hand waarvan verschillende afweercellen gekenmerkt en onderscheiden kunnen worden. De gevolgen die het aantonen van een bepaalde hoeveelheid van deze afwijkende cellen zou hebben voor het type behandeling van RCD patiënten waren ook nog onduidelijk. Daarom was het doel in dit proefschrift om, nadat een overzicht gegeven wordt van de beschikbare literatuur over RCD en EATL (in deel 1):

1. een 'afkapwaarde' (grenswaarde) voor deze afwijkende T-cellen te bepalen, te valideren en te onderbouwen op basis van klinische gegevens met betrekking tot de overleving van RCD en EATL patiënten (hierover gaat deel 2 van dit proefschrift).
2. deze afwijkende T-cellen in de dunne darm verder te karakteriseren en andere factoren te zoeken die betrokken zijn bij het ontstaan van refractaire coeliakie (dit staat beschreven in deel 3).
3. nieuwe therapieën te evalueren, die specifiek gericht zijn tegen deze afwijkende T-cellen, met als doel om de overleving te verbeteren en het ontstaan van lymfeklierkanker te voorkomen of vertragen (dit kunt u lezen in deel 4)

Samenvattend hebben we gevonden dat bij RCD op basis van het aankleuren van de verschillende 'celoppervlakte-markers' op de T-cellen in de dunne darmwand onderscheid gemaakt kan worden tussen RCD type I en II. RCD type I heeft een afkapwaarde van minder dan 20% 'aberrante' T-cellen, RCD type II heeft een afkapwaarde van meer dan 20% 'aberrante' T-cellen. De prognose van refractaire RCD type I (zonder afwijkende cellen) lijkt goed. Sterfte gerelateerd aan deze vorm van coeliakie, of de ontwikkeling van RCD II of EATL, werd niet waargenomen. Daarentegen bestaat er bij de groep patiënten met RCD type II een sterk verhoogde kans op het ontwikkelen van EATL. Binnen 4-6 jaar ontwikkelt 50-60% van de patiënten EATL. De 5-jaarsoverleving is dan slechts 8-20% . EATL is in Nederland een zeldzame aandoening, met 0.10 nieuwe gevallen per 100.000 inwoners per jaar. Bij mensen ouder dan 50 jaar ligt dit aantal aanzienlijk hoger, namelijk 2.08 nieuwe gevallen per 100.000 inwoners per jaar (met name tussen 60 en 70 jaar).
De afwijkende cellen bij RCD II kunnen diffuus verspreid door de darm maar ook buiten de darm en zelfs in de huid gevonden worden. We hebben het moleculaire mechanisme beschreven dat ten grondslag ligt aan het afwijkende markerpatroon van een van de oppervlakte-kenmerken van de 'aberrante' T-cellen bij RCD II, alsmede een genetische factor die een rol lijkt te spelen bij het ontstaan van RCD (MYO9B). Verder is er in het bloed en in de darmwand gekeken naar de aanwezigheid van bepaalde afweercellen die een dempende werking hebben op de ontstekingsreactie. Deze afweercellen, γδ-T-cellen genaamd, bleken met name bij RCD type II verminderd te zijn in de darm, maar wel weer toe te nemen na effectieve therapie zodra de afwijkende cellen afnamen.
De behandeling van RCD blijft een uitdaging, met name wanneer er 'aberrante' T-cellen aanwezig zijn in de darm (RCD type II). De RCD I patiënten reageren goed op therapie die de ontstekingsreactie onderdrukt (Azathioprine en Prednison) en lijken geen verhoogd risico te hebben op EATL. Voor RCD II echter, is momenteel nog geen vaststaande behandeling om EATL te voorkomen. Gelukkig ontwikkelen therapeutische strategieën zich snel. Behandeling specifiek gericht op het uitroeien van de 'aberrante' T-cel populatie in de darm, zoals met Cladribine therapie, is veelbelovend. Verder wordt autologe stamcel transplantatie (ASCT) na hoge dosis chemotherapie verder geëxploreerd, met als doel om de 'ontspoorde' ontstekingsreactie' te doorbreken door de immuunrespons te 'resetten'. Alleen Cladribine en ASCT hebben bewezen

het percentage afwijkende cellen signficant te reduceren. Het uiteindelijke doel van therapie bij RCD II is om EATL te voorkomen of uit te stellen. Want er is momenteel nog geen goede behandeling voor EATL en dit lymfoom heeft een zeer sombere prognose. Omdat het om een zeldzame aandoening met kleine groepen patiënten gaat is optimale internationale samenwerking nodig. Grote studies dienen opgezet te worden, om de verschillende behandelingen te vergelijken en te komen tot een gestandaardiseerde, optimale behandeling voor RCD en EATL patiënten. Voor deze samenwerking zijn duidelijke diagnostische criteria en afspraken nodig, deze zullen hopelijk in 2009 bij het Internationale Coeliakie Congres in Amsterdam vastgesteld gaan worden, zodat de prognose van deze patiënten zal verbeteren.

Part Six
Addendum

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 22 Juli 1980 te Zwolle. Ze groeide op aan zee in Callantsoog en haalde in 1998 haar gymnasium diploma aan het Johannes College in Den Helder. Dat jaar begon zij aan haar studie geneeskunde aan de Vrije Universiteit in Amsterdam, waar zij in 2002 haar doctoraal examen behaalde. Voordat ze aan haar coschappen begon studeerde zij van 2002-2003 kunstgeschiedenis aan de Universiteit van Amsterdam, maakte een reis door Afrika en verbleef enkele maanden in Rome om Italiaans te leren, waarna ze gedurende 4 jaar wekelijks lessen aan het 'Istituto Italiano' in Amsterdam bleef volgen. In 2003 begon ze aan haar co-schappen en in 2005 behaalde ze haar artsexamen. Vervolgens begon ze aan haar wetenschappelijk onderzoek onder begeleiding van Prof.dr.C.J.J. Mulder, bij de afdeling Maag-Darm- Leverziekten in het VU medisch centrum te Amsterdam. Een groot deel van haar onderzoek vond plaats op het Medisch Immunologisch Laboratorium, onder supervisie van Dr.M.W.J. Schreurs en Mw.dr. B.M.E. von Blomberg. In februari 2008 begon ze aan haar opleiding tot Maag-Darm-Leverarts in het St. Lucas Andreas Ziekenhuis in Amsterdam. Momenteel volgt zij haar vooropleiding Interne Geneeskunde (opleider Dr. C.E.H. Siegert) en in 2010 zal ze haar opleiding tot MDL-arts hier vervolgen (opleider Dr. I.C.E. Wesdorp). Ze woont samen met Daan Ten Bosch.

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List of abbreviations

2-CDA	=	2-Chlorodeoxyadenosine; Cladribine
APC	=	Allophycocyan
ASCT	=	Autologous Stem Cell Transplantation
BEAM	=	Carmustine, etoposide, cytarabine, melphalan
BSA	=	Bovine Serum Albumine
CD	=	Coeliac Disease
		Cluster of Differentiation (when a number is indicated)
CHOP	=	Cyclophosphamide, Doxorubicine, Vincristine and Prednisone
CI	=	Confidence interval
CR	=	Complete remission
CT	=	Computed tomography scan
DBE	=	Double Balloon Enteroscopy
DHAP	=	Dexamethasone, Cytarabine and Cisplatinum
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
ENT	=	Ear Nose Throat
EMA	=	anti-endomysium antibodies
EATL	=	Enteropathy Associated T-cell Lymphoma
FACS	=	Fluorescence Activated Cell Scanner
FITC	=	Fluorescein IsoThioCyanate
FoxP3	=	Forkhead box P3
G-CSF	=	Granulocyte-colony stimulating factor
GDS	=	Gastroduodenoscopy
GFD	=	Gluten free diet
GFP	=	Green fluorescent protein
HLA	=	Human Leucocyte Antigen
IEL	=	IntraEpithelial Lymphocyte
IFN-γ	=	Interferon gamma
IL	=	Interleukin
iNKT	=	invariant NKT cells
IMDM	=	Iscove's modified Dulbecco's medium
KGF	=	Keratinocyte Growth Factor
LPL	=	Lamina Propria Lymphocytes
MYO9B	=	Myosin 9B
MRI	=	Magnetic Resonance Imaging
NGFR	=	Nerve growth factor receptor
NHL	=	Non-Hodgkin Lymphoma
NHS	=	Normal human serum
OR	=	Odds ratio
PALGA	=	The nationwide network and registry of histo- and cytopathology in the Netherlands
PBS	=	Phosphate Buffered Saline
PE	=	Phycoerythrin
PerCP	=	Peridin chlorophyll protein
PET	=	Positron Emission Tomography
RCD	=	Refractory Coeliac Disease
TCR	=	T-cell Receptor
TGA	=	anti-tissue transglutaminase antibodies
TGF-β	=	Transforming Growth Factor beta
Tγδ	=	TCR γδ+ cells
Tregs	=	Regulatory T cells
UJ	=	Ulcerative jejunitis
VIM	=	Etoposide, Ifosfamide and Methotrexate
VCE	=	Video Capsule Endoscopy